Generation of MHC class I allospecific regulatory T cells using chimeric antigen receptors, tools for eliciting targeted transplant tolerance

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GENERATION OF MHC CLASS I ALLOSPECIFIC REGULATORY T CELLS USING CHIMERIC ANTIGEN RECEPTORS, TOOLS FOR ELICITING TARGETED TRANSPLANT TOLERANCE

A thesis submitted to the Faculty of Life Sciences and Medicine at King's College London for the degree of Doctor of Philosophy

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ABSTRACT

Regulatory T cells (Treg) therapy using autologous Tregs expanded *ex vivo* is currently being assessed clinically as a means of limiting graft rejection. However, pre-clinical data has demonstrated that graft-specific Tregs protect from graft rejection more effectively than polyclonal Tregs. Chimeric antigen receptor (CAR) technology is currently being investigated clinically as a means of conferring tumour antigen-specificity onto T cells in cancer research. CARs are synthetic fusion proteins which translate the engagement of extracellular target antigens into the activation of intracellular T cell signalling cascades. The hypothesis tested in this thesis was that the efficacy of polyclonal Treg therapy to inhibit transplant rejection could be enhanced by using CARs to confer specificity for donor MHC class I, alloantigens which are ubiquitously expressed in allografts.

A human CAR was constructed to incorporate a patient-derived HLA-A2-targeting moiety and a CD28-CD3ζ signalling domain. Delivery of this CAR into human Tregs which were isolated using GMP-compatible protocols did not influence the phenotype or suppressive capacity of these cells. Compared to polyclonal Tregs, A2 CAR Tregs exhibited a greater suppressive function in the presence of HLA-$A2^+$ antigen presenting cells, without eliciting cytotoxic activity. Furthermore, these cells preferentially transmigrated across HLA-A2-expressing endothelial cell monolayers, suggesting a favoured migration in to HLA-$A2^+$ target tissues. In a human skin xenograft transplant model, A2 CAR Tregs alleviated alloimmune-mediated damage of HLA-$A2^+$ skin more effectively than polyclonal Tregs.

A second CAR was designed to redirect mouse Tregs towards BALB/c MHC class I (K^d) with the rationale of comparing the efficacy of CAR Tregs and Tregs with different allospecificities at reducing graft rejection. Murine CAR Tregs maintained their phenotype and suppressive ability and appeared to proliferate in the presence of K^d *in vivo* although conclusive evidence for the functionality of this CAR remained to be acquired.

The results obtained demonstrated that CARs can be used to generate MHC class I-allospecific Tregs which are functionally superior to polyclonal Tregs at protecting from alloimmune-mediated transplant rejection, suggesting that CAR technology is a clinically applicable refinement of Treg therapy for organ transplantation.

DECLARATION

The work described in this thesis is original research that I have personally carried out, unless stated otherwise. All sources of information are acknowledged by means of reference.

Signature _____ Date _____

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"*Know the true value of time; snatch, seize and enjoy every moment of it. No idleness; no laziness; no procrastination; never put off 'til tomorrow what you can do today*."

Philip Dormer Stanhope, 4th Earl of Chesterfield. 1749

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ABBREVIATIONS

A

aa	Amino acid
ACK	Ammonium chloride potassium (K)
ADP	Adenosine diphosphate
AICD	Activation induced cell death
AIRE	Autoimmune regulator
ALL	Acute lymphocytic leukaemia
AMP	Adenosine monophosphate
AP (protein)	Activator protein
AP (phosphatase)	Alkaline phosphatase
APC (cell)	Antigen presenting cell
APC (fluorochrome)	Allophycocyanin
ARTEMIS	Alloantigen reactive Tregs to facilitate minimisation and/or discontinuation of immunosuppression
ATCC	American type culture collection
ATG	Antithymocyte globulin
ATP	Adenosine triphosphate

B

BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCR	B cell receptor
B-LCL	B-lymphoblastoid cell line
BLT	Bone marrow liver thymus
BM	Bone marrow
BMT	Bone marrow transplantation
bp	Base pair
BSA	Bovine serum albumin
BSU	Biological services unit
BUN	Blood urea nitrogen

C

CAAR	Chimeric autoantibody receptor

cAMP	Cyclic adenosine monophosphate
CAR	Chimeric antigen receptor
CBA	Cytometric bead array
CCR	Chemokine receptor
CCR	Chimeric chemokine receptor
CD	Cluster of differentiation
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CLA	Cutaneous lymphocyte antigen
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CNS	Central nervous system
ConA	Concanavalin A
cpm	Counts per minute
CRISPR	Clustered regularly interspaced short palindromic repeats
CTLA	Cytotoxic T lymphocyte antigen
CTV	CellTrace violet
CXCL	Chemokine (CXC motif) ligand
CXCR	Chemokine (CXC motif) receptor

D

d	Distilled
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DART	Donor-alloantigen-reactive regulatory T cell
DC	Dendritic cell
deLTa	Donor-alloantigen-reactive regulatory T cells in liver transplantation
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
Dsg	Desmoglein

E

E. coli	*Escherichia coli*
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
Eco	Ecotropic
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope

F

FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisation
FCS	Foetal calf serum
FIJI	Fiji is just ImageJ
FITC	Fluorescein
FKBP	FK binding protein
FOXP3/FoxP3	Forkhead box protein 3 (human/mouse)
FRET	Förster resonance energy transfer

G

g	Gram
GAD	Glutamate decarboxylase
GaLV	Gibbon ape leukaemia virus
GITR	Glucocorticoid-induced TNFR-related protein
GJ	Gap junction
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing practice
GOI	Gene of interest
gp	Glycoprotein
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP	Guanosine triphosphate
GvHD	Graft-versus-host disease
Gy	Gray

H

H&E	Hematoxylin and eosin
HA	Hemagglutinin
HBSS	Hank's balanced salt solution
HDR	Homology directed repair
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
HS	Human serum
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HUVEC	Human umbilical vein endothelial cell

I

IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ICOS	Inducible T cell co-stimulator
IDO	Indoleamine dioxygenase
Ig	Immunoglobulin
IHWG	International histocompatibility working group
iIL-12	Inducible IL-12
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IMDPH	Inosine-5′-monophosphate dehydrogenase
Indel	Insertion/deletion
ip	Intraperitoneal
IPEX	Immune deregulatory polyendocrinopathy enteropathy X-linked syndrome
IR	Intra-red
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Induced Treg
iv	Intravenous

J

JAK	Janus kinase

L

L	Litre
L	Ligand
LAG	Lymphocyte activation gene
LB	Lysogeny broth
LFA	Lymphocyte function-associated antigen
LIFT	Lymphocyte immunofluorescence test
LPS	Lipopolysaccharide

M

M	Medium
MART	Melanoma antigen recognised by T cells
MaxIP	Maximum intensity projection
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
miRNA	microRNA
MLR	Mixed leukocyte reaction
MMF	Mycophenolate mofetil
MOG	Myelin oligodendrocyte glycoprotein
MoMuLV	Moloney murine leukaemia virus
MP	Mercaptopurine
MS	Multiple sclerosis
MST	Mean survival time
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex

N

NeuAc	N-Acetylneuraminic acid
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
NHEJ	Non-homologous end joining
NIS	Sodium-iodide symporter
NIS	Nikon imaging software

NK	Natural killer
NOD	Non-obese diabetic
Nrp	Neuropilin
NSG	Non-obese diabetic severe combined immunodeficiency common gamma chain

O

OCT	Optimal cutting temperature
ORF	Open reading frame

P

PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PD	Programmed death
PE	Phycoerythrin
PEG	Polyethylene glycol
PEI	Polyethylenimine
PFHM	Protein-free hybridoma media
PHA	Phytohaemagglutinin
PI3K	Phosphoinositide 3-kinase
PIC	Pre-integration complex
PIFT	Platelet immunofluorescence test
PIL	Procedure individual licence
Pol	Polymerase
PPL	Procedure project licence
PRKDC	Protein kinase, DNA activated, catalytic polypeptide
PRR	Pattern recognition receptor
PSMA	Prostate-specific membrane antigen
PTP	Post-transfusion purpura
pTreg	Peripherally-derived Treg

R

RA	Rheumatoid arthritis

RAG	Recombination-activating gene
Raptor	Regulatory-associated protein of mammalian target of rapamycin
RBC	Red blood cell
RCF	Relative centrifugal force
RECIST	Response evaluation criteria in solid tumours
Rictor	Rapamycin-insensitive companion of mammalian target of rapamycin
RISC	RNA-induced silencing complex
RPMI	Roswell Park Memorial Institute
RTC	Reverse transcription complex

S

scFv	Single-chain variable fragment
SCID	Severe combined immunodeficiency
SLE	Systemic lupus erythematosus
SOC	Super optimal broth with catabolite repression
SOP	Standard operating procedure
SP	Single positive
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
SV40	Simian vacuolating virus 40

T

T1D	Type-I diabetes
TAA	Tumour associated antigen
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TALEN	Transcription activator-like effector nuclease
TASK	Treg adoptive therapy for subclinical inflammation in kidney transplantation
TBI	Total body irradiation
Tconv	Conventional T cell
TCR	T cell receptor
TE	Tris-ethylenediaminetetraacetic acid
TFC	Thyroid follicular cell
TGF	Transforming growth factor
Th	T helper cell
ThRIL	Therapy of regulatory T cells in liver transplantation

TIL	Tumour infiltrating lymphocyte
TILT	T1D immunotherapy using polyclonal Tregs
TK	Thymidine kinase
TLR	Toll-like receptor
TM	Transmembrane
TMB	Tetramethylbenzidine
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
TNP	Trinitrophenol
Tr1	Type-I regulatory T cell
TRACT	Treg adoptive cellular transfer
Treg	Regulatory T cell
TRUCK	T cells redirected for universal cytokine killing
TSDR	Treg specific demethylation region
tTreg	Thymus-derived Treg

U

U	Unit
UVEREG	Uveitis regulatory T cell

V

V(D)J	Variable-diversity-joining
v/v	Volume to volume ratio
V_H	Variable heavy chain
V_L	Variable light chain
VSV-G	Vesicular stomatitis virus protein G

W

w/w	Weight to weight ratio
WR	Working reagent

CHAPTER I

GENERAL INTRODUCTION

1.1 ORGAN TRANSPLANTATION

Improvements in surgical techniques and the development of modern immunosuppressive regimens during the 20th century (Watson and Dark 2012) promoted organ transplantation to become the gold-standard treatment option for treating the dysfunction and failure of various organs (Sagoo, Lombardi et al. 2012, Safinia, Leech et al. 2013). However, the therapeutic benefits of this treatment option are limited by graft rejection, a complex process by which recipient immune cells recognise and destroy transplanted tissues, ultimately resulting in allograft dysfunction and failure (Rogers and Lechler 2001, Tsang, Tanriver et al. 2009, Booth, Grabauskiene et al. 2011, Sagoo, Lombardi et al. 2012, Pasquet, Douet et al. 2013).

Graft rejection is currently managed with life-long drug-based immunosuppression which has facilitated improvements in short-term survival rates (Safinia, Leech et al. 2013). However, these approaches are non-specific and as such, associated with severe side-effects: recipients are under consistent nephrotoxic insult with an increased susceptibility to acquiring infections and developing cancer. More importantly, these approaches do not effectively tackle the issue of chronic rejection, thus the half-life of allografts remains limited to approximately 10-15 years (Burgos, Gonzalez-Molina et al. 2012, Gruessner and Gruessner 2013). This compounds another limitation of organ transplantation which relates to a shortage of available donor organs (Testa and Siegler 2014).

In light of these issues, various approaches are currently under investigation as potential means of inducing a state of "transplant tolerance", whereby transplanted tissues are accepted indefinitely, negating the requirement for life-long immunosuppression.

1.1.1 PATHWAYS OF ALLORECOGNITION

Although the innate immune response has been shown to contribute to graft rejection (Oberbarnscheidt and Lakkis 2014), studies conducted in neonatally thymectomised (Miller 1961) and irradiated adult (Hall, Dorsch et al. 1978, Hall, Dorsch et al. 1978, Hall, de Saxe et al. 1983) mice have demonstrated that the most deleterious immune responses are driven by recipient-derived T cells. These cells have been described to recognise alloantigens via three pathways of allorecognition: the direct, indirect and semi-direct pathways. The direct pathway is initiated by donor-derived antigen presenting cells (APC) which present intact allogeneic major histocompatibility complex (MHC)-peptide complexes to recipient T cells (Figure 1-1A). Conversely, the indirect pathway relies on recipient-derived APCs which uptake, process and

present allopeptides in the context of self-MHC class II (Figure 1-1B). More recently, the semi-direct pathway was described in which recipient-derived APCs present both acquired, intact allo-MHC-peptide complexes (direct presentation) and allopeptides in the context of self-MHC (indirect presentation) (Figure 1-1C).

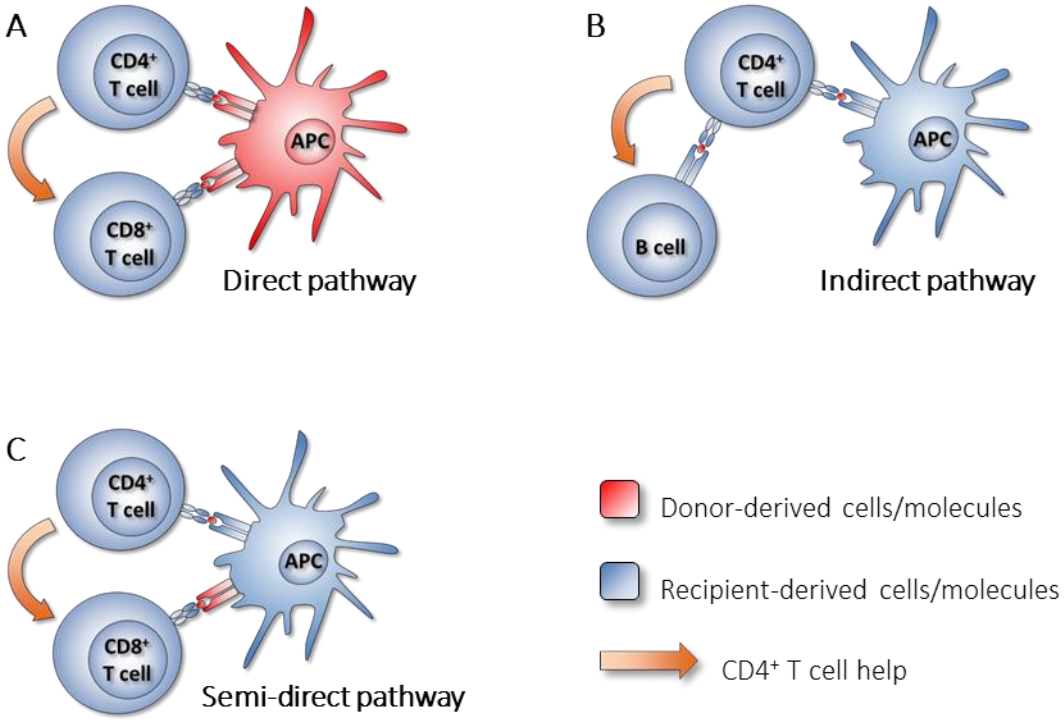

Figure 1-1 | **Schematic diagram detailing the three distinct pathways of allorecognition.** *A: In the direct pathway of allorecognition, donor APCs migrate from the transplanted allograft to secondary lymphoid organs where they present donor antigen in the context of donor MHC molecules. Recipient CD4⁺ T cells binding allopeptides presented by allo-MHC class II are then capable of facilitating the activation of recipient CD8⁺ T cells recognising allopeptides presented by allo-MHC class I. B: In the indirect pathway of allorecognition, recipient APCs infiltrate the transplanted allograft where they acquire donor antigen. These APCs then migrate to secondary lymphoid organs where they present these allopeptides in the context of self-MHC class II to recipient CD4⁺ T cells which, in turn, can facilitate the activation of recipient B cells. C: In the semi-direct pathway of allorecognition, recipient APCs infiltrate the transplanted allograft and uptake intact donor class I and class II MHC/peptide complexes. In addition, these APCs also uptake, process and present peptides derived from donor MHC molecules in the context of self-MHC class II. Therefore, in the secondary lymphoid organs, these recipient APCs are capable of presenting allopeptides in the context of self-MHC class II to recipient CD4⁺ T cells (indirect) and in the context of allo-MHC class I to recipient CD8⁺ T cells (direct), enabling the CD4⁺ T cells to facilitate in the activation of the CD8⁺ T cells.*

1.1.1.1 Direct pathway of allorecognition

A review article pertaining to the direct pathway of allorecognition is included at the end of this thesis.

One of the most striking features of the direct pathway is the strength and vigour with which a subsequent immune response is elicited. This was first demonstrated *in vitro* through the use of mixed leukocyte reactions (MLR) (Bain, Vas et al. 1964) and subsequently *in vivo* using allogeneic rodent transplant models (Sherman and Chattopadhyay 1993). This vigour was attributed to the presence of donor-derived "passenger" leukocytes which are co-transferred into the recipient, during a transplant procedure. Indeed, depletion of these cells from thyroid (Talmage, Dart et al. 1976) or pancreatic (Bowen, Andrus et al. 1980) allografts has been shown to prolong allograft survival. In 1982, Lechler and Batchelor (Lechler and Batchelor 1982, Lechler and Batchelor 1982) demonstrated that the principle passenger leukocytes responsible for activating recipient T cells were dendritic cells (DC). In these studies, prolonged survival of rat kidney allografts could be achieved by "parking" the allograft in an intermediate recipient to deplete passenger leukocytes, prior to engraftment in a terminal recipient. However, this prolongation was prevented by the repletion of donor DCs.

The vigour of a direct alloimmune response can be attributed to the fact that individuals have a disproportionately high precursor frequency of T cells with direct allospecificity. Approximately 1-10% of the T cells in an individual are capable of engaging an intact foreign MHC-peptide complex. This is orders of magnitude greater than the ~0.01% of T cells which typically recognise foreign peptides presented in the context of self-MHC (Veerapathran, Pidala et al. 2011). Two models have been proposed to explain this unusually high precursor frequency, each of which places an emphasis on one of the components which comprises an MHC-peptide complex: the allopeptide and the allo-MHC molecule.

1.1.1.1.1 *Models explaining direct allorecognition*

The "multiple binary complexes" model suggests that allo-MHC molecules "mimic" specific structural components of self-MHC molecules, specifically the components which are recognised by an imposing T cell receptor (TCR) (Matzinger and Bevan 1977). However, as the binding groove of the allo-MHC differs greatly from the self-MHC molecule, the pool of peptides naturally presented is different, despite being derived from similar endogenous proteins. As such, in this model it is the allopeptide presented which drives direct allorecognition (Figure 1-2A). In support of this, Eckels *et al.* (Eckels, Gorski et al. 1988) demonstrated in 1988 that T cell proliferation

induced by the presentation of allopeptides in the context of human leukocyte antigen (HLA)-DR1 was abrogated when the allopeptides were displaced by competing influenza haemagglutinin-based peptides. It has also been shown that presentation of allo-MHC molecules

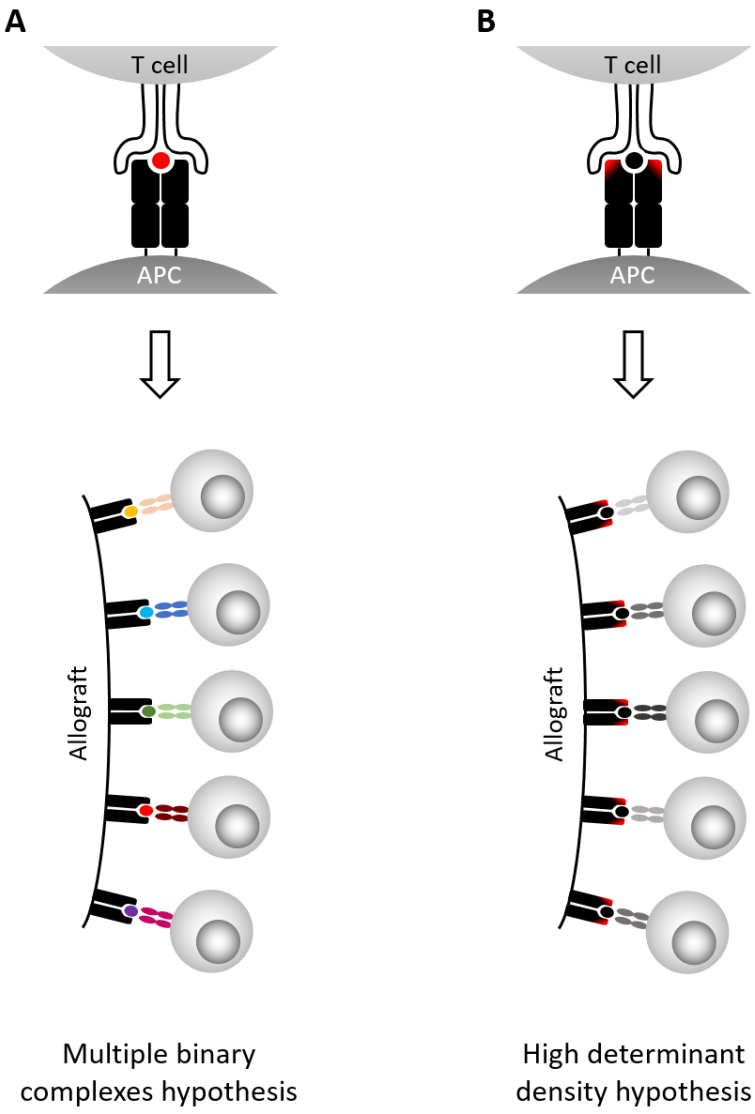

Figure 1-2 | **Comparison of the two principle theories explaining the high frequency of T cells with direct allospecificity.** *A: Multiple binary complexes hypothesis. The elements of the allogeneic MHC molecule which interact with the TCR mimic those which are found in self-MHC molecules. As such, it is the presence of the allopeptide (red) which drives recognition of the allogeneic MHC-peptide complex. Allograft presentation of various allopeptides in the contexts of MHC molecules which are perceived as 'self' results in the activation of a range of T cells, each expressing a TCR specific for a different MHC-peptide complex. B: High determinant density hypothesis. Structural differences in the polymorphic regions of the allo-MHC molecule are detected by the TCR (red). The high density of cognate allo-MHC molecules which possess these polymorphisms on donor-derived APCs facilitates the efficient activation of recipient T cells which recognise the allogeneic MHC molecule with a low, medium or high affinity.*

lacking allopeptides, achieved through the use of MHC mutants (Heath, Kane et al. 1991) or acid-treatment of target cells (Wang, Man et al. 1998), trigger limited responses from alloreactive T cells.

The "high determinant density" model focuses on the fact that allo-MHCs are structural different to self-MHCs (Bevan 1984). Whilst the majority of elements recognised by a TCR are conserved across various MHC subtypes (Huseby, White et al. 2005), specific amino acid polymorphisms present in the exposed parts of the allo-MHC molecule modify the manner by which a self-restricted TCR docks with an MHC, irrespective of the peptide presented. These residues cause the MHC-peptide complex to be recognised as foreign, thus the peptide presented stabilises the MHC-peptide complex but has little influence in the recognition process. The high density of ligands expressed by donor APCs can further facilitate the activation of alloreactive T cells (Figure 1-2B). Additionally, the affinity with which this TCR:allo-MHC interaction occurs may have implications in the alloresponse observed. Whilst T cells are selected to bind self-MHC-peptide complexes with a low affinity, it is possible that they would bind allo-MHC-peptide complexes with a high affinity, suggesting that a high affinity cross-reaction is responsible for the allorecognition observed. In support of this model, Lombardi *et al.* demonstrated that site directed mutagenesis of specific TCR contact regions within allogeneic HLA-DR molecules resulted in inhibition of T cell binding and subsequent effector responses (Lombardi, Barber et al. 1991). As such, it was evident that specific sites of the allo-MHC molecule were critical for direct allorecognition to occur. These findings were later confirmed by Villadangos *et al.* who employed a similar approach whilst mutating HLA-B27 (Villadangos, Galocha et al. 1994).

1.1.1.1.2 *Conundrum of direct allorecognition*

It is well-established that T cell progenitors must undergo a stepwise "education" process in the thymus to develop into mature T cell. The cells which typically result from this process are able to recognise self-MHC-peptide complexes with a low affinity (discussed in section 1.1.3.1). However, the existence of this process reveals a conundrum: why do self-restricted T cells (Zinkernagel and Doherty 1974) recognise allo-MHC-peptide complexes? Studies have demonstrated that cross-reactivity between self and allogeneic MHC-peptide complexes is key for this mode of allorecognition. In other words, T cells specific for peptide "x" presented by self-MHC "A" are also able to recognise peptide "y" presented by allo-MHC "B" (Game and Lechler 2002).

Studies supporting this have demonstrated that lymphocyte function-associated antigen (LFA)-3$^+$ (Lombardi, Sidhu et al. 1990) and CD45RO$^+$ (Merkenschlager, Ikeda et al. 1991) memory T cells primed against peptide "x" presented by self-MHC "A" also respond to allo-MHC-peptide complexes (peptide "y" presented by allo-MHC "B"). Furthermore, these cross-reacting memory T cells comprise a significant proportion of the total T cells which respond in a direct manner. This cross-reactivity concept was further accentuated by Lombardi *et al.* in 1989 (Lombardi, Sidhu et al. 1989). In this study, human alloreactive T cell clones which were specific for HLA-DR1 were co-cultured with autologous APCs presenting *Candida albicans*-derived antigens in the context of HLA-DR4/HLA-DR13. Half of the alloreactive T cell clones analysed responded in these co-cultures, suggesting that cells which were capable of recognising the allo-MHC molecule HLA-DR1 were also activated by APCs presenting peptides in the context of self-MHC.

Overall, the aforementioned high determinant density and multiple binary complex models provide two explanations for why T cells with direct allospecificity exist with a high precursor frequency. With functional and structural data supporting both hypotheses (Boardman, Jacob et al. 2016), it is likely that *in vivo*, the high frequency of direct allorecognition can be attributed to a combination of these theories.

1.1.1.1.3 *Consequences of direct allorecognition*

Allorecognition typically leads to an effector response in which CD8$^+$ T cells with direct allospecificity actively kill donor-derived target cells (Yu, Xu et al. 2006), leading to allograft dysfunction and failure. Various studies have investigated how recipient graft-specific CD8$^+$ and CD4$^+$ T cells contribute to acute and chronic transplant rejection (Rosenberg and Singer 1992, Lee, Grusby et al. 1994, Krieger, Yin et al. 1996, Game and Lechler 2002, Benichou, Yamada et al. 2011). For example, in 2000, Pietra *et al.* investigated the contribution of CD4$^+$ T cells in acute graft rejection through the use of severe combined immunodeficiency (SCID) and recombination-activating-gene (RAG)-1 deficient mice which lack functional T and B cells (Pietra, Wiseman et al. 2000). C57BL/6 heart allografts, which survived indefinitely in SCID mice, were acutely rejected (mean survival time (MST) of 12 days) when BALB/c CD4$^+$ T cells were adoptively transferred on the day of transplant. Conversely, heart allografts which lacked donor C57BL/6 MHC class II molecules (C2D donor mice) were not rejected (7/8 allografts survived >60 days), demonstrating that CD4$^+$ T cells directly specific for donor MHC class II molecules were necessary for acute allograft rejection.

More recently, Brown *et al.* further demonstrated the contribution of recipient $CD4^+$ T cells to allograft rejection using a fully-mismatched kidney transplant model in which donor APCs were specifically depleted (Brown, Nowocin et al. 2016). In this study, one native kidney of recipient mice (FVB strain) was replaced with an allogeneic (C57BL/6×CBA F1) kidney. In the absence of treatment, transplanted kidneys were rejected acutely in 40% of cases. However, in recipients which were treated with an immunotoxin-conjugated antibody specific for donor MHC class II ($I\text{-}A^k$) to depleted donor APCs, kidney allografts were completely protected: histological analysis showed no evidence of rejection and upon removal of the second native kidney, the function of the transplanted kidney (blood urea nitrogen (BUN) score) was found to be intact.

Analysis of blood samples acquired from stable renal transplant recipients has revealed that recipient $CD4^+$ T cells with direct allospecificity become hyporesponsive towards alloantigens but are not deleted (Ng, Baker et al. 2001). This work was extended by demonstrating that that human $CD4^+$ T cells co-cultured with MHC class II-expressing thyroid follicular cells (TFC) (Lombardi, Arnold et al. 1997) or epithelial cells (Marelli-Berg, Weetman et al. 1997) do not proliferate or produce cytokines in the absence of co-stimulation and are hyporesponsive upon subsequent challenge with Epstein-Barr virus (EBV)-transformed Lymphoblastoid B cell lines (B-LCL) (Marelli-Berg and Lechler 1999). Together, these results suggested that in the absence of donor-derived professional APCs, recipient $CD4^+$ T cells engaged MHC class II molecules presented by transplanted tissue parenchymal cells which lacks co-stimulatory molecules. As a result, these T cells become anergic (Marelli-Berg, Weetman et al. 1997) or polarised towards a Th2 phenotype (Lombardi, Arnold et al. 1997), suggesting that prolonged alloimmune responses depend on an alternative mode of allorecognition.

1.1.1.2 Indirect pathway of allorecognition

The indirect pathway was originally proposed as a distinct “route” of alloimmunisation by Lechler and Batchelor *et al*. in 1982 as part of the aforementioned rat kidney “parking” studies (Lechler and Batchelor 1982, Lechler and Batchelor 1982). In these studies, it was noted that although depletion of donor DCs prolonged the survival of rat kidney allografts, rejection still occurred, albeit at a slower rate compared to allografts which contained allogeneic DCs. This led to the proposal that alloreactive T cells were activated via a second pathway of allorecognition which did not rely on donor-derived APCs (Game and Lechler 2002).

The MHC locus is one of the most polymorphic regions of the genome in vertebrates (Sommer 2005). Consequently, graft rejection is believed to principally be driven through the recognition

of donor MHC molecules, either in an intact form (direct allorecognition) or as peptides presented in the context of recipient-MHC molecules (indirect allorecognition). In the early 1990s, various studies indeed demonstrated that recipient APCs were able to present peptides derived from donor-MHC molecules and that this process contributed to allograft rejection (Benichou, Takizawa et al. 1992, Fangmann, Dalchau et al. 1992, Liu, Sun et al. 1993, Benham, Sawyer et al. 1995). At this time, it was also estimated that the frequency of T cells with indirect allospecificity prior to transplantation was approximately 100-fold lower than T cells with direct allospecificity (Liu, Sun et al. 1993), a finding which has subsequently been confirmed on numerous occasions (Veerapathran, Pidala et al. 2011). As such, the intensity of this alloimmune response was not believed to be as vigorous as a direct alloimmune response (Benichou, Takizawa et al. 1992) but the consistent availability of donor antigens in the allograft meant that this pathway could persist long-term. Consequently, the indirect pathway was proposed to be the main mode of chronic allograft rejection (Hornick and Lechler 1997).

The first experimental evidence for the contribution of the indirect pathway to graft rejection was provided by Auchincloss *et al*. in 1993 (Auchincloss, Lee et al. 1993). In this study, skin grafts from MHC class II knockout mice were transplanted onto recipient mice which were depleted of $CD4^+$ or $CD8^+$ T cells. A key finding was that MHC class II-deficient skin was rejected in recipients which were depleted of $CD8^+$ T cells. This attributed an important role to $CD4^+$ T cells in the rejection process. Furthermore, it suggested that these $CD4^+$ T cells were primed by recipient APCs presenting allopeptides, not donor APCs as these cells were MHC class II-deficient. The importance of these $CD4^+$ T cells to aid the activation of $CD8^+$ T cells was subsequently demonstrated by Lee *et al*. in 1994 using the same model (Lee, Grusby et al. 1994).

1.1.1.2.1 *Cross-presentation*

The studies described above principally demonstrated how $CD4^+$ T cells with indirect allospecificity contributed to allograft rejection. However, it is now clear that $CD8^+$ T cells with indirect allospecific can also be activated. This relies on recipient APCs presenting acquired peptides in the context of recipient MHC class I, a process termed “cross-presentation”. Although the concept of cross-presentation was first demonstrated in 1976 (Bevan 1976, Bevan 2006), the implications of this for allograft rejection were not realised for many years. In 2002, Valujskikh *et al*. demonstrated that recipient $CD8^+$ T cells which were cross-primed by recipient APCs contributed to graft rejection in an IFNγ-dependent manner (Valujskikh, Lantz et al. 2002). However, the relative contribution of these $CD8^+$ T cells with indirect allospecificity to ultimate allograft rejection may be limited (Valujskikh, Zhang et al. 2006).

1.1.1.3 Semi-direct pathway of allorecognition

As alluded to above, donor antigens acquired from exogenous origins are naturally presented in the context of MHC class II. Consequently, it is primarily $CD4^+$ T cells which recognise alloantigens in an indirect manner, not $CD8^+$ T cells which are typically responsible for eliciting cytotoxicity. Furthermore, efficient $CD8^+$ T cell activation requires help from $CD4^+$ T cells (Krieger, Yin et al. 1996). As such, it is reasonable to conclude that a link between the direct and indirect pathways of allorecognition exists and that $CD4^+$ T cells with indirect allospecificity facilitate the activation of $CD8^+$ T cells with direct allospecificity (Lee, Grusby et al. 1994, Lee, Grusby et al. 1997). Theoretically, for this to occur, two separate APCs should be present: a recipient APC presenting allopeptides indirectly to the $CD4^+$ T cell and a donor APC presenting antigen directly to the $CD8^+$ T cell. However, from a practical viewpoint, it is highly unlikely that these two APC:T cell interactions naturally occur in such close proximity, leading to a conundrum termed the "four-cell conundrum". This was resolved by the discovery of APCs which present intact donor-MHC-peptide complexes in a direct manner and allopeptides in an indirect manner (Herrera, Golshayan et al. 2004, Jiang, Herrera et al. 2004, Smyth, Harker et al. 2008), the basis of a more recently described third pathway of allorecognition: the semi-direct pathway (Herrera, Golshayan et al. 2004).

1.1.1.3.1 *Cross-dressing*

In a similar manner to the indirect pathway, the semi-direct pathway relies on recipient-derived APCs which infiltrate the allograft. However, in addition to presenting allopeptides indirectly, these cells also acquire intact donor MHC-peptide complexes from donor-derived cells/tissues, a phenomenon termed "cross-dressing". This allows recipient APCs presenting allopeptides indirectly to also present allogeneic MHC-peptide complexes in a direct manner (Smyth, Harker et al. 2008).

Indeed, recipient DCs presenting both donor MHC class I-peptide complexes (direct presentation) and allopeptides in the context of self-MHC (indirect presentation) have been observed in various contexts including following kidney (Brown, Sacks et al. 2011) and heart (Sivaganesh, Harper et al. 2013, Harper, Ali et al. 2015) transplantation. Through this pathway, direct alloimmune responses can continue long after donor-derived APCs have died, but the extent to which this pathway contributes to allograft rejection is not yet known. Recently, the host laboratory has shown that after removal of the direct pathway and in the absence of cross-presentation, acquired allo-MHC-peptide complexes on recipient DCs can drive allograft rejection throughout the life-span of the transplant (Smyth, Lechler et al. 2016).

1.1.2 IMMUNOSUPPRESSION

Organ transplantation would not be a viable treatment option if approaches had not been developed to reduce the effects of graft rejection. Immunosuppression is an approach which been explored extensively over the past century as a potential means of solving this problem (Barker and Markmann 2013).

In 1914, working on the hypothesis that tumour xenograft rejection was a consequence of recipient lymphocyte activation, Murphy demonstrated that irradiation of recipient rats using a low dose of x-rays facilitated the prolonged survival of mouse-derived tumours (Murphy 1914, Murphy 1914). However, the significance of these findings was not truly appreciated until 1955, when Main and Prehn demonstrated that radiation of adult mice weakened the immune system to an extent that allowed for the engraftment of allogeneic bone marrow cells (Main and Prehn 1955). Importantly, resultant chimeric mice were able to accept skin grafts which were transplanted from the same donor as the bone marrow cells (Barker and Markmann 2013). These findings proposed that immunosuppression through irradiation could prolong allograft survival, thus this concept was quickly translated to the clinic. In the late 1950s and early 1960s, kidney transplant procedures between non-identical twins were performed where recipients were pre-conditioned with total body irradiation (TBI). These procedures were a resounding success whereby the two recipients survived for 20 and 26 years post-transplant (Barker and Markmann 2013).

In 1962, Küss and Hamburger began to explore the benefits of coupling chemical-based immunosuppression with TBI in humans (Kuss, Legrain et al. 1962). Using 6-mercoptopurine (6-MP), which had previously been demonstrated to prolong the survival of skin allografts in rabbits (Schwartz and Dameshek 1960), and adrenal cortical steroid-based immunosuppression in addition to TBI, Küss *et al.* performed two kidney transplant procedures between non-related patients and achieved long-term survival (>9 months) in both cases. Studies performed over the following decades saw TBI superseded by various immunosuppressive drugs including the cytotoxic drug “azathioprine” (Murray, Merrill et al. 1963), the non-cytotoxic fungal derivative “cyclosporine A” (Calne, Rolles et al. 1979), and the powerful anti-inflammatory drug “prednisone” (Starzl, Weil et al. 1980), the latter two of which are still used today.

1.1.2.1 Current immunosuppressive drugs in transplantation

Modern immunosuppressive regimens principally target lymphocytes due to their particularly deleterious contribution to allograft rejection. To achieve this, a variety of drugs are used in combination to target different pathways involved with lymphocyte activation/proliferation. Although the precise drugs used varies between institution, the regimen adopted will typically include a combination of calcineurin inhibitors (CNI), corticosteroids, anti-proliferative agents and potentially mammalian target of rapamycin (mTOR) inhibitors. For example, the standard drugs prescribed for kidney transplant recipients at Guy's Hospital are tacrolimus (a CNI inhibitor), prednisone (a corticosteroid) and mycophenolate mofetil (MMF; an anti-proliferative agent).

CNIs function by inhibiting calcineurin, a fundamental serine-threonine phosphatase involved in T cell activation (Hamawy 2003). Typically, TCR engagement causes an increase in the concentration of intracellular Ca^{2+} which results in the activation of calcineurin (Yamashita, Katsumata et al. 2000). Calcineurin then activates nuclear factor of activated T cells (NFAT) which is important for transcribing key genes including *interleukin* (*IL*)*-2* (Hogan, Chen et al. 2003). Two drugs are commonly used to inhibit this process in transplantation: cyclosporine A and the bacterial derivative tacrolimus. These drugs function by forming complexes with immunophilins, a family of intracellular cis-trans peptidyl-prolyl isomerases (Marks 1996). Cyclosporine A binds to cyclophilins and tacrolimus binds to the 12 kDa FK-binding protein (FKBP12) (Hamawy 2003). The complexes formed bind calcineurin which in turn inhibits the action of calcineurin and ultimately prevents activation of the T cell. Rapamycin and everolimus inhibit T cell signalling in a similar manner to these drugs but instead of targeting calcineurin, these drugs inhibit mTOR. The use of rapamycin and its relation with regulatory T cell (Treg) therapy is discussed in Chapter IV.

Corticosteroids such as prednisone are steroid hormones which function by inhibiting the activation of numerous pro-inflammatory genes in a wide variety of cells (Barnes 2006). To achieve this, corticosteroids readily diffuse across cell membranes and bind gluorcortocoid receptors (GR) in the cytoplasm. "Active GRs" can then translocate into the nucleus where they encourage an anti-inflammatory state through a variety of mechanisms. Active GRs can promote the expression of specific target genes by binding glucocorticoid response elements (GRE) (Dostert and Heinzel 2004). Alternatively, active GRs interact with, and inhibit, pro-inflammatory transcription factors such as activator protein (AP)-1 and nuclear factor (NF)-κB, thus indirectly suppress the synthesis of cytokines, inflammatory enzymes, adhesion molecules and inflammatory receptors (Barnes 2006).

Anti-proliferative drugs are used to inhibit the growth of T and B lymphocytes. In the past, this was achieved through the use of cytotoxic drugs such as cyclophosphamide. However, due to

severe side-effects, these drugs have been replaced with azathioprine and MMF (Ostraat, Qi et al. 1997, Meier-Kriesche, Morris et al. 2004, Clayton, McDonald et al. 2012). Azathioprine is a purine analogue which blocks purine synthesis and incorporates into replicating DNA, preventing successful replication (Maltzman and Koretzky 2003). Similarly, MMF blocks inosine monophosphate dehydrogenase (IMPDH) thus prevents the synthesis of guanine nucleotides required for DNA replication (Fulton and Markham 1996).

All three of the drug categories described above reduce the rate of allograft rejection but concurrently elicit numerous side effects. CNIs are associated with neurotoxicity (Anghel, Tanasescu et al. 2013), nephrotoxicity (Fioretto, Najafian et al. 2011) and development of diabetes (van Hooff, Christiaans et al. 2004), with up to 20% of non-renal transplant recipients developing chronic renal failure after using CNIs for 5 years (Ojo, Held et al. 2003). Corticosteroids have a particular influence on the endocrine system, thus can cause a wide variety of side effects including osteoporosis, gain of weight and changes in mental state (e.g. aggression, agitation, depression and anxiety) (Buchman 2001, Barnes 2006) whilst anti-proliferative drugs primarily result in gastrointestinal side effects such as nausea and diarrhoea (Stolk, Boerbooms et al. 1998, Martinez, Nos et al. 2001, Ginzler, Dooley et al. 2005). These side effects are coupled with the inherent effects caused by immunosuppression, leaving recipients with an increased susceptibility to acquiring infections and developing cancer (Niethammer, Kummerle-Deschner et al. 1999, Bustami, Ojo et al. 2004).

1.1.2.2 Induction therapy and drug weaning

Intensive immunosuppression is often given over the first few months following a transplant procedure as this is when the risk of acute graft rejection is highest (Brennan, Daller et al. 2006, Kirk 2006). However, as this risk diminishes over time, the immunosuppression prescribed is typically reduced to a life-long “maintenance” level, such that the potency of the accompanying side-effects is limited (Turner and Knechtle 2013).

The therapy provided during the initial months following the transplant procedure is of particular importance for the long-term outcome of the allograft. As such, additional immunosuppressive agents may be used at this time as part of an “induction therapy” protocol and weaned over the forthcoming months. These additional agents are often antigen-specific antibodies which target lymphocytes, in particular T cells. Examples include muromonab-CD3 (OKT3 clone), antithymocyte globulin (ATG; horse/rabbit-derived antibodies specific for human T cells), IL-2 receptor-specific antibodies (declizumab or basiliximab) and alemtuzumab, an antibody specific

for CD52 which is expressed on mature lymphocytes (Rosenberg, Vriesendorp et al. 2005, Goldfarb-Rumyantzev, Smith et al. 2006, Watson and Dark 2012).

This induction therapy period is of particular importance when considering the use of alternative therapeutic approaches such as Treg therapy (Scotta, Fanelli et al. 2016) which is described below (section 1.2.4).

1.1.3 Mechanisms of Tolerance

Immunological tolerance is a state in which an immune response is not initiated towards an antigen which would normally be expected to provoke a response. Tolerance towards self-antigens is essential to prevent the development of autoimmune diseases. The ultimate goal of research in the transplant field is to induce a state of tolerance towards allogeneic antigens, thereby negating the requirement for immunosuppression. Mechanisms which prevent the development and maturation of autoreactive cells are referred to as "central tolerance" mechanisms. Autoreactive cells which escape these mechanisms are often suppressed by "peripheral tolerance" mechanisms. These two branches of tolerance are discussed in detail below.

1.1.3.1 Central Tolerance

Central tolerance mechanisms are implemented in primary lymphoid organs, namely the bone marrow (BM) for B cells and the thymus for T cells. In these organs, common lymphoid progenitor (CLP) cells undergo variable-diversity-joining (V(D)J) recombination to generate a B cell receptor (BCR) or TCR with a given specificity. This process is almost entirely random and as such, the receptor generated has the potential to recognise self-antigens with a high affinity. Central tolerance mechanisms are in place to delete autoreactive lymphocytes before they develop into immunocompetent cells.

In the thymus, T cell progenitors, or "thymocytes", must undergo two selection processes before they can mature into T cells. The first of these is termed "positive selection" which ensures that the TCR presented by the thymocyte is capable of recognising peptides presented in the context of self-MHC molecules. Thymocytes which do not meet this requirement are neglected and undergo programmed cell death (PCD). Surviving thymocytes then undergo a "negative selection" process whereby cells presenting a TCR which binds self-MHC-peptide complexes with a high

affinity are deleted. This latter process is aided by medullary stromal cells which express the *autoimmune regulator* (*AIRE*) gene, allowing for the presentation of tissue-specific antigens which would otherwise not be expressed in the thymus. The majority of cells which survive these processes are able to recognise self-MHC-peptide complexes with a low affinity and are released into the periphery as naïve T cells. However, a small proportion of the $CD4^+$ thymocytes which recognise self-MHC-peptide complexes with a higher than average affinity are induced to express the transcription factor Forkhead box protein 3 (FOXP3) and commit to the Treg lineage. These cells are described in detail in section 1.2.

1.1.3.2 Peripheral Tolerance

Although lymphocytes undergo rigorous selection processes, it is possible for autoreactive cells to escape negative selection and to be released into the periphery. The potentially deleterious effects of these cells are managed by mechanisms of peripheral tolerance which act at numerous levels, as described below.

1.1.3.2.1 *Induction of anergy*

For naïve T cells to be successfully activated, three signals must be received. "Signal 1" is provided by the TCR complex as a result of the TCR engaging a cognate MHC-peptide complex. During this process, CD4 or CD8 co-receptors from the T cell make additional contacts with the MHC molecule, restricting $CD8^+$ T cells to recognise peptides presented in the context of MHC class I and $CD4^+$ T cells to recognise peptides presented in the context of MHC class II. In rare cases, this restriction can be overcome if a TCR engages an MHC-peptide complex with a particularly high affinity (Plesa, Zheng et al. 2012).

"Signal 2" is a co-stimulatory signal which is provided by various proteins present in an immunological synapse (IS). The paradigmatic co-stimulatory signal is delivered by CD28 which typically engages CD80 or CD86, co-stimulatory molecules present on the surface of the APC. Additional co-stimulatory signals can be provided by 4-1BB (CD137), OX40 (CD134), inducible T cell co-stimulator (ICOS; CD278), CD30 and CD40 ligand (CD40L) which engage 4-1BBL, OX40L (CD252), ICOSL (CD275), CD153 and CD40, respectively. The latter molecules are present on APCs (Acuto and Michel 2003) which have been activated as a consequence of signalling through pattern recognition receptors (PRR). As such, co-stimulatory molecules are not expressed by the majority of cells in the periphery. Delivery of signal 1 without signal 2 induces a state of

unresponsiveness in T cells, known as a state of "anergy", which may proceed active deletion of the T cell. Consequently, autoreactive T cells which engage self-MHC-peptide complexes presented by peripheral tissues are rendered anergic, thus cannot undergo clonal expansion and initiate an immune response.

"Signal 3" is provided by the detection of polarising cytokines. These signals are most relevant for determining the subtype which the T cell will differentiate into. However, mechanisms which disrupt cytokine detection, particularly IL-2 signalling which is essential for T cell proliferation and differentiation, can greatly influence the maturation of T cells. This is of relevance to a mechanism by which $CD25^+$ Tregs function, as described below (sections 1.1.3.2.4 and 1.2.3).

1.1.3.2.2 *Activation-induced cell death*

Activation-induced cell death (AICD) is a natural consequence of T cell activation and clonal expansion. It ensures that T cell clones diminish in number following clearance of a target antigen and facilitates in the elimination of autoreactive T cells (Waring and Mullbacher 1999). T cells naturally express Fas (CD95) and following TCR engagement, upregulate the expression of Fas ligand (FasL; CD178) (Zhang, Xu et al. 2004). Consequently, apoptosis is induced in T cell clones through the Fas/FasL pathway in an autocrine manner (Dhein, Walczak et al. 1995). Indeed, mice lacking either Fas (homozygous *lpr*) or FasL (homozygous *gld*) suffer from uncontrolled lymphoproliferation and autoimmunity (Watanabe-Fukunaga, Brannan et al. 1992, Takahashi, Tanaka et al. 1994). Similarly, apoptosis induced through Bim and Bcl-2/Bcl-xL-dependent permeabilisation of the outer mitochondrial membrane has also been demonstrated to be important for efficient deletion of autoreactive lymphocytes (Marrack and Kappler 2004, Mueller 2010, Xing and Hogquist 2012). As a tolerance mechanism, AICD results in deletion of autoreactive T cell clones which are activated in the periphery.

1.1.3.2.3 *Immunological ignorance*

Efficient lymphocyte activation inherently requires the lymphocyte to engage with its target antigen. However, in certain scenarios, this is not possible due to physical constrictions (Mueller 2010). A prime example is the blood-brain barrier (BBB) which severely limits lymphocyte trafficking into the central nervous system (CNS) (Engelhardt 2006). As such, lymphocytes which may be autoreactive and recognise a target antigen in the brain are segregated from their target antigen and consequently not activated.

1.1.3.2.4 *Suppression*

Suppression of autoreactive T cell activation in the periphery is a key mechanism of tolerance which is principally enforced by Tregs. Indeed, depletion of Tregs, achieved either through targeted killing (Kim, Lahl et al. 2009) or mutations in the *FoxP3* locus (Brunkow, Jeffery et al. 2001), has been shown to cause severe and often fatal autoimmunity. Tregs mediate the activation of target cells, including autoreactive lymphocytes, using a plethora of different contact dependent and independent mechanisms, as discussed below (section 1.2.3).

1.2 REGULATORY T CELLS

The idea of cells suppressing and regulating the immune system has been around for many years. In 1969, Nishizuka and Sakakura published the first experimental evidence which suggested the existence of thymus-derived cells which were capable of suppressing autoimmunity in mice (Nishizuka and Sakakura 1969). These findings were confirmed by Gershon and Kondo in the early 1970s in a series of publications which culminated in the coinage of the term "suppressor T cell". However, a shadow of scepticism was cast over this research area when subsequent studies pertaining to a key I-J suppressor molecule were proved wrong. This scepticism was accentuated by the lack of a definitive suppressor T cell marker and limited data relating to the mechanisms employed to suppress target cells. As such, despite the discovery of the *FoxP3* gene in 1991, interest in suppressive lymphocytes was not reignited until 1995 when Sakaguchi *et al*. published a seminal paper describing a population of $CD4^+CD25^+$ T cells which were capable of inhibiting autoimmunity upon adoptive transfer. In this study, the authors showed that the onset of autoimmunity in athymic nude mice injected with $CD4^+CD25^-$ T cells was hindered by the co-injection of $CD4^+CD25^+$ T cells. Furthermore, they showed prolonged survival of skin allografts following adoptive transfer of $CD4^+CD25^+$ T cells. Over the years that followed, various studies demonstrated the existence of $CD4^+CD25^+$ Tregs in humans, suggesting an important role for these cells in the maintenance of tolerance *in vivo* (Dieckmann, Plottner et al. 2001, Ng, Duggan et al. 2001, Taams, Smith et al. 2001, Taams, Vukmanovic-Stejic et al. 2002).

1.2.1 REGULATORY T CELL SUBSETS

Subsequent studies proceeded to demonstrate that murine $CD4^+CD25^+$ T cells were a unique lineage of T cells (Suri-Payer, Amar et al. 1998) which were capable of suppressing undesired immune responses in a plethora of different contexts ranging from organ transplantation

(Kingsley, Karim et al. 2002, Golshayan, Jiang et al. 2007, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013) to various autoimmune diseases (Kohm, Carpentier et al. 2002, Mottet, Uhlig et al. 2003, Tang, Henriksen et al. 2004, Zheng, Wang et al. 2004, Morgan, Flierman et al. 2005). Although the definition of what constitutes a Treg subset is a matter of debate, it is widely accepted that multiple subtypes of Tregs exist. At the most fundamental level, Tregs can be split into three distinct groups: $CD4^+$ Tregs which are derived from the thymus (tTregs), $CD4^+$ T cells which are induced in the periphery (pTregs) or *in vitro* (iTregs) to exhibit a suppressive function and suppressive $CD8^+$ T cells. A brief description of these suppressive T cell subtypes is provided below.

1.2.1.1 Thymus-derived Tregs

Colloquial references to "regulatory T cells" can stereotypically be interpreted as references to tTregs which were previously termed "naturally-occurring Tregs". tTregs constitute approximately 5-10% of all peripheral $CD4^+$ T cells (Tang, Henriksen et al. 2004, Tsang, Tanriver et al. 2009) and develop from a proportion of thymocytes which recognise self-antigens with a comparatively high affinity. In the thymus, these cells are induced to constitutively expressed the lineage-determining transcription factor FOXP3. In mice, mutations in the *FoxP3* gene has been shown to induce a "scurfy" phenotype which is characterised by scaly/ruffled skin, reddened eyes, secondary lymphoid organ enlargement and premature death (Ramsdell and Ziegler 2014). Similarly, *FOXP3* mutations in humans have been directly linked with the severe autoimmune disease immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), accentuating the critical role of these cells *in vivo* (Bennett, Christie et al. 2001). In 2009, Miyara *et al*. delineated three phenotypically and functionally distinct subpopulations of human tTregs which were differentiated by their expression of FOXP3 and CD45RA (Miyara, Yoshioka et al. 2009): $CD45RA^+FOXP3^{lo}$ resting Tregs, $CD45RA^-FOXP3^{hi}$ activated Tregs and $CD45RA^-FOXP3^{lo}$ T cells which secreted cytokines but were non-suppressive.

1.2.1.2 Periphery-derived and *in vitro*-induced Tregs

The nomenclature relating to T cells which are induced to be suppressive can be confusing. Tregs which are derived *in vivo* in the periphery are termed pTregs. However, cells which are generated under similar conditions *ex vivo* are termed induced Tregs (iTregs) (Abbas, Benoist et al. 2013, Shevach and Thornton 2014, Guo and Zhou 2015).

Although not as well characterised as tTregs, pTregs have been implicated with the induction and maintenance of tolerance, mostly through the secretion of immunosuppressive cytokines. It is believed that at least two types of pTregs exist, namely Type-I regulatory T cells (Tr1) and T helper 3 cells (Th3) which secrete high levels of IL-10 (Roncarolo, Bacchetta et al. 2001) and transforming growth factor (TGF)-β, respectively (Weiner 2001). Tr1 cells were initially described in 1997 and characterised by their ability to suppress colitis (Groux, O'Garra et al. 1997) whilst Th3 cells were first identified in 1994 by their role in oral tolerance induction in a multiple sclerosis (MS) context (Chen, Kuchroo et al. 1994). Both of these cell types are now primarily associated with mucosal immunity, as demonstrated by the clinical application of Tr1 cells in the treatment of Crohn's disease (Desreumaux, Foussat et al. 2012). However, the inherent instability of these cells is a cause for concern (Chen, Kim et al. 2011, Shevach and Thornton 2014, Kanamori, Nakatsukasa et al. 2016).

Over the years, various groups have performed microarrays on tTregs and iTregs to identify genes which are differentially expressed between these two cell types. From these studies, two molecules were proposed as potential markers of tTregs: Helios (*Ikzf2*) and neuropilin-1 (Thornton, Korty et al. 2010, Yadav, Louvet et al. 2012). However, subsequent analyses have demonstrated that not all tTregs express these markers thus their use to delineate tTregs is controversial (Singh, Hjort et al. 2015, Elkord 2016, Kanamori, Nakatsukasa et al. 2016).

1.2.1.3 Suppressive $CD8^+$ T cells

In a similar manner to $CD4^+$ Tregs, murine studies have demonstrated that $CD8^+$ Tregs can be derived naturally or induced (Pomie, Menager-Marcq et al. 2008). In rodents, at least four types of suppressive $CD8^+$ T cell have been described, although the exact nature and phenotype of these cells varies between studies: $CD8^+CD25^+FOXP3^+CD28^-$ T cells (Najafian, Chitnis et al. 2003, Churlaud, Pitoiset et al. 2015), $CD8^+Qa\text{-}1^+$ T cells (Hu, Ikizawa et al. 2004), $CD8^+CD122^+CD49^{lo}$ T cells (Akane, Kojima et al. 2016) and $CD8^+CD45RC^{lo}$ T cells (Xystrakis, Dejean et al. 2004). Mouse-based studies have demonstrated the suppressive capacity of $CD8^+$ Tregs for preventing experimental autoimmune encephalomyelitis (EAE) (Najafian, Chitnis et al. 2003, Hu, Ikizawa et al. 2004, Lee, Ishida et al. 2008) and colitis (Menager-Marcq, Pomie et al. 2006), with an important role attributed to the secretion of suppressive cytokines (Pomie, Menager-Marcq et al. 2008). Recently, Bézie *et al.* demonstrated that rodent and human $CD8^+CD45RC^{lo}$ Tregs produced IL-34 which was shown to induce transplant tolerance in a rat cardiac allograft model (Bezie, Picarda et al. 2015). Together, these findings reflected observational studies performed in humans which correlated low numbers of $CD8^+$ Tregs (particularly $CD8^+CD25^+FOXP3^+$ T cells) with an increased

incidence of systemic erythematosus (SLE) (Filaci, Bacilieri et al. 2001, Tulunay, Yavuz et al. 2008), inflammatory bowel disease (IBD) (Brimnes, Allez et al. 2005) and MS (Frisullo, Nociti et al. 2010, Baughman, Mendoza et al. 2011).

Given the principle focus of this thesis, henceforth descriptions of "Tregs" are references to thymic-derived tTreg cells, unless stated otherwise.

1.2.2 Regulatory T cell phenotype

Over the past 20 years, various markers have been suggested to define Tregs. However, this has developed into a particularly complex field and to date, has not alluded a definitive Treg marker. As such, Tregs are often described using a combination of different markers (Corthay 2009). A description of the most importance markers is provided below.

1.2.2.1 CD25

In 1995, Sakaguchi *et al*. described CD25 as a fundamental marker of Tregs in mice (Sakaguchi, Sakaguchi et al. 1995). However, as CD25 is the α chain of the IL-2 receptor and IL-2 is a critical T cell growth factor, this marker is transiently upregulated on activated conventional T cells (Tconv) in both mice and humans. As such, CD25 is not an exclusive marker of Tregs. This is complicated further in humans as conventional T cells can express an intermediate level of CD25, necessitating Tregs to be defined as $CD25^{hi}$. Despite these caveats, the constitutively high expression of CD25 on Tregs makes it a useful marker for enriching these cells for a variety of *in vitro* applications and as such, enrichment of $CD25^{+}$ cells is currently used to isolate Tregs for clinical use. The function of CD25 with relation to a Treg mechanism of action is discussed in section 1.2.3.2.

1.2.2.2 FoxP3

Various studies in 2003 revealed FoxP3 as the "master regulator" of Tregs (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Khattri, Cox et al. 2003). In mice, FoxP3 is almost exclusively expressed in Tregs and as such, it is a suitable marker for defining these cells. However, this situation is more complicated in humans as FOXP3 is upregulated in human $CD4^{+}CD25^{-}$ Tconvs following activation. Currently, one of the only approaches which exists for identifying *bona fide* Tregs is to analyse the methylation state of the Treg specific demethylation region (TSDR) which

is located in the *FOXP3* gene. The TSDR is demethylated in Tregs but methylated in Tconvs although the methylation state of the TSDR cannot be used to isolate clinically-applicable Tregs as the cells must be lysed to perform this analysis.

1.2.2.3 CD127

Although TSDR analysis provides a conclusive means of defining Tregs *in vitro*, the cell lysis required for this analysis means that it cannot be employed for isolating Tregs for clinical use. Similarly, FOXP3 expression cannot be employed as this requires the cells to be permeabilised. To facilitate the isolation of Tregs for clinical use, it was necessary to identify a cell surface marker which was differentially expressed in Tregs and activated $CD25^+$ Tconvs. This was achieved in 2006 when Liu *et al.* demonstrated that CD127 expression was inversely correlated with FOXP3 expression. CD127 is the IL-7 receptor α chain and is commonly used in combination with CD4 and CD25 expression to define human Tregs for various *in vitro* assays. Furthermore, $CD4^+CD25^{hi}CD127^{lo}$ Tregs are currently isolated by cell sorting as the cell product for various clinical trials outside of the UK.

1.2.2.4 Helios

In 2010, Thornton *et al*. described Helios, a member of the Ikaros transcription factor family, as a key molecule which was differentially expressed in tTregs and pTregs (Thornton, Korty et al. 2010). However, a plethora of subsequent studies disputed this claim, demonstrating that Helios, which was supposedly tTreg specific, could be induced in pTregs and that $Helios^+$ and $Helios^-$ Tregs existed within a standard human tTreg pool. Despite this somewhat sketchy history, Helios expression defines a stable population of Tregs with an enhanced suppressive capacity, compared to $Helios^-$ Tregs, although the precise mechanisms as to how this is achieved remain unsolved.

1.2.2.5 CD45RA

CD45RA is an isoform of CD45 which is primarily expressed on naïve T cells. Following TCR activation, an alternative splicing approach is adopted for CD45 such that CD45RA expression is downregulated and CD45RO expression is upregulated (Zikherman and Weiss 2008). Consequently, naive T cells are often defined as $CD45RA^+CD45RO^-$ whilst memory T cells are $CD45RA^-CD45RO^+$. CD45RA expression can define a population of naïve Tregs which constitutively

express CD25 and/or FOXP3, circumventing the aforementioned issues regarding the inadvertent isolation of activated Tconvs. In 2006, Hoffmann *et al*. demonstrated that $CD4^+CD25^{hi}CD45RA^+$ Tregs were highly suppressive cells which maintained constitutive expression of FOXP3 in contrast to $CD4^+CD25^{hi}CD45RA^-$ cells which were moderately suppressive and lost FOXP3 expression following *in vitro* expansion (Hoffmann, Eder et al. 2006). This work was expanded on by Miyara *et al*. who used the expression of CD45RA in combination with FOXP3 expression to define three subpopulations of tTregs (section 1.2.1.1). From this study, population III, which consisted of cells defined as $CD4^+CD25^+FOXP3^{lo}CD45RA^-$, was of particular interest as these cells were shown to secrete cytokines not elicit suppression (Miyara, Yoshioka et al. 2009). However, this finding is controversial as unpublished data from the host laboratory and Prof Irma Joosten (Radboud University Medical Center, Nijmegen, Netherlands) has suggested that population III Tregs may in fact possess a suppressive ability but at a reduced level to that exhibited by other Treg populations.

1.2.2.6 HLA-DR

HLA-DR is the most prevalent HLA class II molecule expressed in humans. Along with the HLA class I molecules HLA-A and HLA-B, particular efforts are made to match the alleles of the HLA-DR locus between donors and recipients in a transplant setting to minimise graft rejection. In 2006, Baecher-Allan *et al*. used HLA-DR expression on Tregs to distinguish two Treg subpopulations: $HLA\text{-}DR^+$ cells which elicited an early (within 72 hours) suppressive response, primarily in a contact-dependent manner, and $HLA\text{-}DR^-$ cells which elicited an early response through the secretion of immunosuppressive cytokines, such as IL-10 (Baecher-Allan, Wolf et al. 2006).

1.2.2.7 Co-stimulatory and co-inhibitory receptors

As previously discussed (section 1.1.3.2.1), successful activation of T cells requires multiple signals including co-stimulatory signals. In a similar manner to Tconvs, activation and expansion of Tregs requires the engagement of co-stimulatory signals. Certain members of the tumour necrosis factor (TNF) receptor superfamily are selectively expressed on Tregs in the absence of immune activation including OX40, GITR, TNFR2 and CD27 although their expression is upregulated in Tconvs upon activation. Interestingly, higher CD27 expression by human Tregs has also been correlated with a greater suppressive potency (Koenen, Fasse et al. 2005).

Treg expression of co-inhibitory molecules is a particularly important mechanism which is employed for suppressing target cells. Cytotoxic T lymphocyte antigen (CTLA)-4 is a potent co-inhibitory receptor which is expressed constitutively on Tregs. Similarly, programmed death (PD)-1 receptor is also expressed at high levels on Tregs (Baecher-Allan, Brown et al. 2003). Both of these molecules are members of the immunoglobulin (Ig) superfamily and are upregulated on Tconvs upon activation. However, in contrast to the aforementioned co-stimulatory molecules, these receptors transmit inhibitory signals into the T cell upon engagement. In Tregs, CTLA-4 employs numerous different approaches to elicit a regulatory function, as discussed in section 1.2.3.

1.2.2.8 Homing receptors

The *in vivo* trafficking of Tregs is an important feature of these cells which is primarily dictated through the expression of different homing receptors. Although not exclusive to Tregs, the expression profile of homing receptors is commonly used to define Tregs, particularly in humans. Naïve CD45RA$^+$ Tregs typically express a range of lymphoid tissue homing receptors such as CD62L, chemokine receptor (CCR)7 and CXCR4 (Hoffmann, Eder et al. 2006) whilst the homing receptors expressed by memory Tregs is dependent on the initial site of activation for each cell. For Tregs expanded *ex vivo*, the presence of specific drugs can have a dramatic effect on the range of homing receptors expressed. Tregs expanded in the presence of retinoic acid express high levels of gut homing receptors such as CCR9 and integrin $\alpha_4\beta_7$ whilst Tregs expanded in the presence of rapamycin express more skin homing receptors such as CCR4, CCR6, CCR10 and cutaneous lymphocyte antigen (CLA) (Scotta, Esposito et al. 2013). Additional homing receptors may also be considered for characterising Tregs including CCR2, CCR5, CXCR3, CXCR5, CXCR6 (Campbell and Koch 2011, Duhen, Duhen et al. 2012).

1.2.2.9 Functional markers

In addition to the various markers described above, Tregs are often characterised by their expression of functional markers such as CTLA-4, CD39, CD73, glucocorticoid-induced TNF receptor (GITR), lymphocyte-activation gene (LAG)3 and Nrp-1. A detailed description of these molecules is provided in section 1.2.3.

1.2.3 REGULATORY T CELL MECHANISMS OF SUPPRESSION

Tregs function to maintain tolerance *in vivo* by suppressing a wide range of target cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer (NK) cells and APCs such as DCs. In order to accomplish this, Tregs employ a range of contact dependent and contact independent mechanisms (Sojka, Huang et al. 2008, Lee, Kim et al. 2011). With new mechanisms consistently being discovered, it is difficult to comprehensively describe how Tregs function but it is safe to assume that a combination of these approaches must be employed to maintain tolerance *in vivo*. A non-exhaustive description of the mechanisms Tregs employ is provided below.

1.2.3.1 Mechanisms influencing APC activation of T cells

Efficient activation of naïve T cells typically requires stimulation by professional APCs such as DCs. Tregs employ numerous mechanisms to disrupt this process and hinder the activation of T cells (Vignali, Collison et al. 2008, Corthay 2009, Sakaguchi, Wing et al. 2009, Shevach 2009). This is paradigmatically achieved through the high expression of CTLA-4 which, as mentioned in section 1.2.2.7, elicits a regulatory function through a number of approaches. CTLA-4 binds the B7 co-stimulatory molecules CD80 and CD86 on the surface of APCs with a high affinity, compared to CD28. This allows CTLA-4 to outcompete CD28, consequently sequestering the ability of the APC to activate Tconvs (Walunas, Bakker et al. 1996). CTLA-4 is also capable of reverse-signalling through CD80/CD86 and upregulating indoleamine 2,3-dioxygenase (IDO) in DCs which, as described below, has an immunoregulatory effect (Grohmann, Orabona et al. 2002, Fallarino, Grohmann et al. 2003). However, the most eminent mechanism by which CTLA-4 functions was discovered in 2011 when it was demonstrated that CTLA-4 could facilitate the physical removal of CD80 and CD86 from the cell surface of APCs (Qureshi, Zheng et al. 2011), a process termed trans-endocytosis (Figure 1-3A). Consequently, without these fundamental co-stimulatory molecules, APCs inadvertently induce anergy in target T cells instead of activating them.

Various other molecules are expressed on the surface of Tregs and, in a similar manner to CTLA-4, function to decrease the ability of APCs to activate T cells. Nrp-1 expressed by Tregs binds intercellular adhesion molecule (ICAM)-1 on the surface of DCs with a high affinity which causes the DCs to interact with the Tregs for a prolonged period of time (Figure 1-3B). During this period, the DCs are less able to activate Tconvs which may be in the vicinity (Sarris, Andersen et al. 2008). Alternatively, LAG3 expressed by Tregs can bind MHC class II with a high affinity and induce immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory signalling pathways in DCs (Figure 1-3C). This signalling causes the DCs to become resistant to maturation

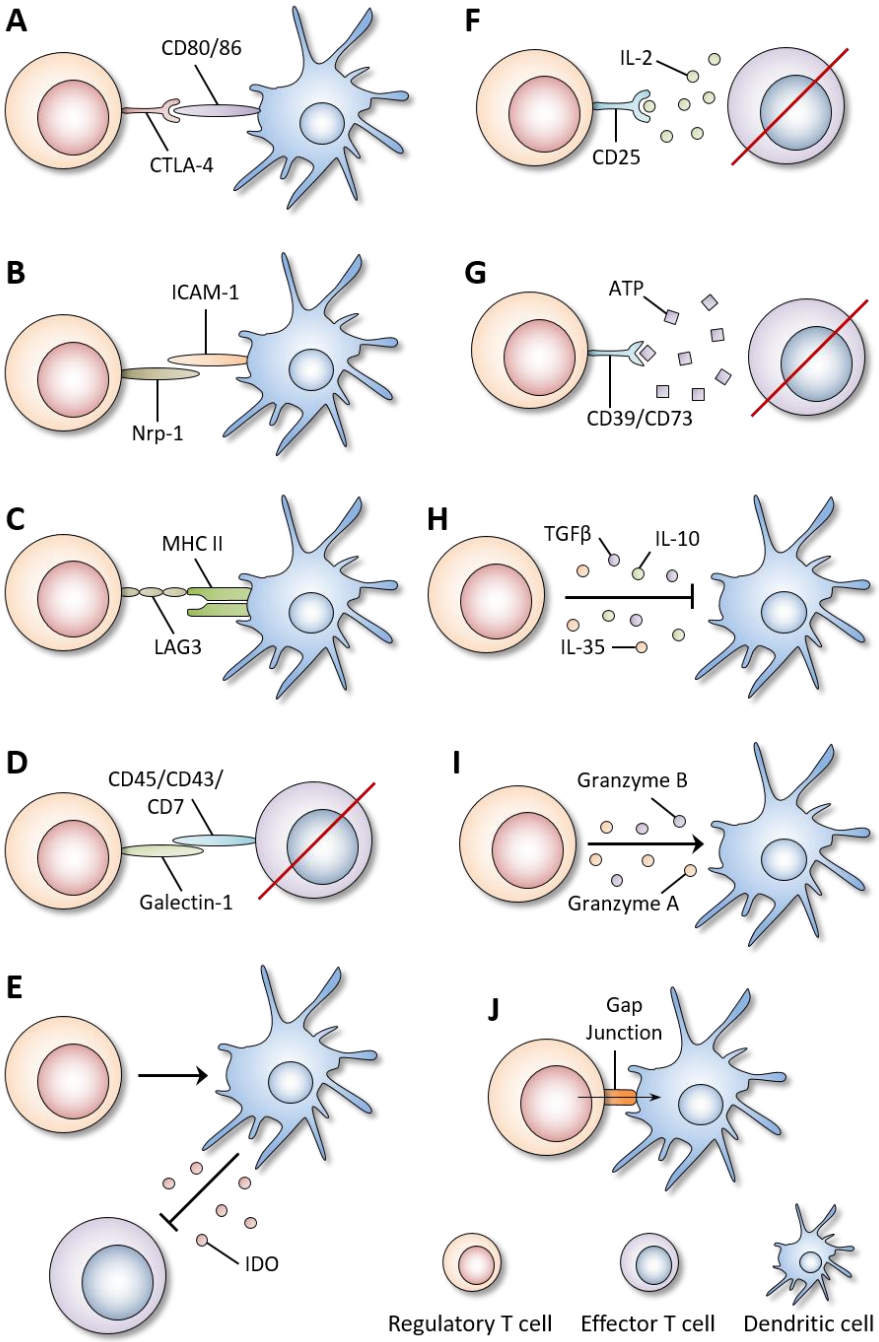

Figure 1-3 | **Mechanisms of Treg suppression adapted from (Shevach 2009) and (Vignali, Collison et al. 2008).** *A: CTLA-4 expressed by Tregs binds CD80/CD86 on the DC surface with a high affinity enabling the Tregs to remove these co-stimulatory molecules from the DC membrane (Qureshi, Zheng et al. 2011). B: Nrp-1 binds ICAM-1 present on the DC surface extending the interaction time with the Treg thus decreasing the length of time available for Tconv activation by the DC (Sarris, Andersen et al. 2008). C: LAG3 binding of MHC class II induces inhibitory intracellular signalling within a DC via SHP-1 which results in inhibition of DC maturation (Liang, Workman et al. 2008). D: Binding of CD45/43/7 by galectin-1 induces growth cycle arrest and apoptosis of activated Teffs (Garin, Chu et al. 2007). E: Tregs can induce DCs to produce IDO which catabolises tryptophan thus suppresses T cell proliferation (Mellor and Munn 2004). F: CD25 binding of IL-2 deprives Tconvs of this necessary T cell growth factor (de la Rosa, Rutz et al. 2004). G: ATP hydrolysis by CD39/73 produces adenosine which inhibits T cell function (Borsellino,*

Kleinewietfeld et al. 2007). H: Tregs can secrete inhibitory cytokines to suppress DC maturation/function (Shevach 2009). I: Tregs can use granzymes to kill target cells utilising a similar mechanism to NK cells (Cao, Cai et al. 2007). J: Small molecules including cAMP can be intercellularly transferred via gap junctions into the cytoplasm of DCs to target specific processes within DCs as a mechanism of regulation (Bopp, Becker et al. 2007).

(LPS-induced upregulation of CD86) and have a decreased capacity to activate T cells (Liang, Workman et al. 2008).

As previously mentioned, DC expression of IDO can have an immunoregulatory effect on T cells. IDO is an enzyme which catabolises indole-containing molecules, such as tryptophan, into kynurenines (Mellor and Munn 2004). This consequently depletes the local environment of tryptophan, an essential amino acid required for T cell proliferation (Figure 1-3E). Furthermore, the increase in kynurenine production also has a suppressive effect on T cell (Terness, Bauer et al. 2002).

1.2.3.2 Mechanisms directly affecting T cells

In addition to inhibiting T cell activation indirectly through manipulating APCs, Tregs can also directly suppress T cells. One mechanism is elicited by galectin-1 which is expressed on Tregs. Galectin-1 binds glycoproteins present on the surface of target T cells, such as CD7, CD43 and CD45 (Figure 1-3D). Through this binding, Tregs can induce cell cycle arrest and/or apoptosis in the target T cell (Garin, Chu et al. 2007). Similarly, PD-L1 engagement of PD-1 on the surface of target T cells triggers inhibitory signalling pathways in T cells, preventing T cell activation and proliferation (Baecher-Allan, Brown et al. 2003).

Tregs also manipulate their microenvironment to suppress local T cells in a contact-independent manner. Through expressing high levels of CD25, Tregs are able to bind IL-2 in the vicinity and deprive local T cells of this crucial growth factor (de la Rosa, Rutz et al. 2004) (Figure 1-3F). Furthermore, Tregs also express CD73 and CD39 (Figure 1-3G). These are ectoenzymes which function together to facilitate the conversion of pro-inflammatory adenosine triphosphate (ATP) into the anti-inflammatory adenosine: CD39 hydrolyses ATP/adenosine diphosphate (ADP) into adenosine monophosphate (AMP) (Borsellino, Kleinewietfeld et al. 2007, Yegutkin 2008) and CD73 catalyses the conversion of AMP into adenosine (Antonioli, Pacher et al. 2013).

1.2.3.3 Mechanisms affecting APCs and T cells

Certain mechanisms employed by Tregs can affect both the function of APCs and T cells. These mechanisms tend to rely on the secretion of immunuoregulatory molecules or vesicles which influence local cells. The most established means by which this is achieved is through the secretion of immunosuppressive cytokines (Figure 1-3H). Following activation, Tregs secrete high levels of IL-10, TGFβ and IL-35 (Levings, Bacchetta et al. 2002, Shevach 2009, Francisco, Sage et al. 2010) which are known to suppress a wide range of immune cells including APCs and T cells (Del Prete, De Carli et al. 1993, Yoshimura and Muto 2011). Tregs also secrete microvesicles called exosomes to exert a suppressive function. These exosomes can facilitate the intercellular transfer of a variety of molecules including surface proteins and cytoplasmic molecules including microRNAs. In 2013, Smyth *et al.* demonstrated that murine Tregs secreted exosomes which contained functional CD73 and had an immunosuppressive capacity (Smyth, Ratnasothy et al. 2013). Treg-derived exosomes were subsequently shown to also facilitate the intercellular transfer of microRNAs as a mechanism of suppression (Okoye, Coomes et al. 2014). Tregs can also exert a suppressive function though eliciting cytotoxicity towards target cells. It has been demonstrated that Tregs are capable of secreting granzymes and perform (Figure 1-3I) in a similar manner to $CD8^+$ T cells and NK cells and this has been used to kill $CD4^+$ T cells, $CD8^+$ T cells and monocytes (Grossman, Verbsky et al. 2004, Grossman, Verbsky et al. 2004, Cai, Cao et al. 2010) as well as tumour cells (Cao, Cai et al. 2007).

In addition to the secretion of suppressive species, Tregs can also inhibit target cells in a contact-dependent manner through the intercellular transfer of immunoregulatory molecules. One mechanism involves the formation of gap junctions (GJ) between Tregs and target cells (Figure 1-3J) (Ring, Karakhanova et al. 2010). GJ are specialised channels which provide a direct cytoplasmic link between cells, allowing the intercellular transfer of small molecules (Evans and Martin 2002). To date, Tregs have been shown form GJs with DCs (Ring, Karakhanova et al. 2010) and T cells (Bopp, Becker et al. 2007). In the latter study, Tregs elicited a suppressive function over T cells by transferring the secondary messenger cyclic (c)AMP. However, it is possible that alternative regulatory molecules may also be transferred via GJs such as microRNAs (Zong, Zhu et al. 2016).

1.2.4 Regulatory T cell therapy

A wide range of studies have noted a link between the proportion of Tregs *in vivo* and tolerance induction. This was most pertinently observed in human transplant recipients where a clear

correlation between the number of Tregs in a graft and allograft survival has been made (Louis, Braudeau et al. 2006, Safinia, Leech et al. 2013). To apply these findings in a clinical setting, two approaches can be taken to increase the number of Tregs *in vivo*: i) to use drugs, compounds or cells (such as tolerogenic DCs) which preferentially expand Tregs *in situ* (Giannoukakis, Phillips et al. 2011, Klawitter, Nashan et al. 2015), or; ii) to infuse a population of Tregs expanded *ex vivo* (Bluestone 2005, Sagoo, Ali et al. 2011, Singer, King et al. 2014, Safinia, Vaikunthanathan et al. 2016).

As detailed above, various immunosuppressive drugs are used following a transplant procedure to limit graft rejection (section 1.1.2.1) including mTOR inhibits such as rapamycin and everolimus (Klawitter, Nashan et al. 2015) which facilitate the preferential expansion of Tregs (Singh, Horvath et al. 2009, Lu, Qian et al. 2010). Furthermore, clinical trials have investigated the safety and efficacy of low dose IL-2 therapy in Type I diabetes (T1D) (Hartemann, Bensimon et al. 2013, Rosenzwajg, Churlaud et al. 2015), SLE (Humrich, von Spee-Mayer et al. 2015) and graft-versus-host disease (GvHD) (Kennedy-Nasser, Ku et al. 2014) as a means of promoting the expansion of Tregs *in vivo*. A limited therapeutic efficacy was observed in the T1D patients but more promising results were obtained in the SLE and GvHD patients. Recently, success has also been achieved in the preferential expansion of Tregs *in vivo* through the use of IL-2/anti-IL-2 complexes which can be manipulated such that they are selectively recognised by Tregs *in vivo*. In 2013, Kim *et al*. demonstrated in mice that renal ischemia reperfusion injury could be attenuated by the administration of IL-2/anti-IL-2 complexes prior to injury induction (Kim, Koo et al. 2013). Similarly, Webster *et al.* demonstrated the efficacy of IL-2/anti-IL-2 complexes at protecting against EAE and rejection of islet allografts in mice (Webster, Walters et al. 2009). In both studies, protection was shown to be mediated through the expansion of Tregs. However, it has also been demonstrated that this Treg promotion is not sufficient for allowing engraftment of BM transplants in mice whilst adoptive Treg therapy is, calling into question the ultimate therapeutic potential of these approaches (Mahr, Unger et al. 2016).

1.2.4.1 Pre-clinical studies of regulatory T cell therapy

The potential for Treg therapy using $CD4^+CD25^+$ Tregs was initially demonstrated by Sakaguchi *et al*. in 1995 (Sakaguchi, Sakaguchi et al. 1995). As previously mentioned (section 1.2), this study showed that the onset of autoimmune diseases in athymic nude mice injected with $CD4^+CD25^-$ T cells was hindered by the co-injection of $CD4^+CD25^+$ T cells. Furthermore, the authors demonstrated prolonged survival of skin allografts following adoptive transfer of $CD4^+CD25^+$ T cells. Following on from this seminal paper, various groups demonstrated the potential for

$CD4^+CD25^+$ Treg therapy in a variety of animal models including GvHD (Taylor, Lees et al. 2002), organ transplantation (Kingsley, Karim et al. 2002), MS (Kohm, Carpentier et al. 2002), colitis (Mottet, Uhlig et al. 2003), lupus erythematosus (Zheng, Wang et al. 2004), T1D (Tang, Henriksen et al. 2004) and rheumatoid arthritis (RA) (Morgan, Flierman et al. 2005).

In a solid tissue transplant context, Treg therapy has been shown to significantly prolong the survival of skin (Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009, Smyth, Lechler et al. 2016), kidney (Brown, Sacks et al. 2011), heart (Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009, Sivaganesh, Harper et al. 2013, Harper, Ali et al. 2015) and islet (Xiao, Ma et al. 2014) allografts. In these studies, the specificity of the Tregs was shown to be of particular importance and significantly influenced the efficacy with which the Tregs protected the allografts.

1.2.4.1.1 *Importance of antigen-specificity for Treg function*

As alluded to above, the efficacy of Treg therapy can be improved by using Tregs with a defined antigen-specificity. This is supported by a substantial number of studies conducted in pre-clinical animal models which have demonstrated that Tregs specific for a desired antigen are functionally superior to polyclonal Tregs (Taylor, Lees et al. 2002, Tang, Henriksen et al. 2004, Joffre, Santolaria et al. 2008, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013).

One of the first studies to compare the efficacy of antigen-specific and self-specific Tregs upon adoptive transfer *in vivo* was conducted by Taylor *et al.* in the context of GvHD (Taylor, Lees et al. 2002). In addition to being one of the first papers to explore the potential for Treg therapy using *ex vivo*-expanded Tregs, this study also demonstrated that murine Tregs preferentially expanded using allogeneic splenocytes were more effective at protecting from GvHD than Tregs expanded using anti-CD3, upon adoptive transfer. In 2004, Tang *et al.* demonstrated a similar superior efficacy of antigen-specific Tregs using a T1D model (Tang, Henriksen et al. 2004). In this study, T1D induced by the transfer of $25x10^6$ diabetogenic cells was blocked by the transfer of $2x10^6$ Tregs specific for islet β-cell antigens, but not as efficiently blocked by the transfer of $8x10^6$ self-specific Tregs, suggesting that the antigen-specific Tregs were over four times more potent at preventing disease manifestation. Furthermore, these antigen-specific Tregs could not only prevent the development of T1D but were also able to reverse diabetes following disease onset, further accentuating the potency of these cells for clinical use.

In a transplant context, numerous studies have demonstrated Tregs with direct allospecificity enhance allograft survival more effectively than self-specific Tregs. In most cases, these cells are

obtained using a preferential expansion approach where Tregs are stimulated with allogeneic APCs (Joffre, Santolaria et al. 2008, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009). These findings have been confirmed in a human setting using a human skin xenograft transplant model (sections 5.1 and 5.3.3.2). In these latter studies, human Tregs were expanded using allogeneic DCs (Sagoo, Ali et al. 2011) or B cells (Putnam, Safinia et al. 2013) and demonstrated a superior efficacy at protecting human skin allografts from alloimmune-mediated skin injury compared to polyclonal Tregs. However, the expansion of Tregs with indirect allospecificity has proved more challenging due to the low frequency of these cells naturally present in the periphery (Veerapathran, Pidala et al. 2011).

Antigen-specific Tregs may also be generated by transducing Tregs to express specific α and β TCR chain genes. The superior efficacy of TCR-modified antigen-specific Tregs, as compared to self-specific Tregs, has been demonstrated in mouse models for the treatment of colitis (Zhou, Borojevic et al. 2004), MS (Stephens, Malpass et al. 2009), autoimmune gastritis (DiPaolo, Brinster et al. 2007), RA (Fujio, Okamoto et al. 2006, Wright, Notley et al. 2009), T1D (Tang, Henriksen et al. 2004, Tarbell, Petit et al. 2007), GvHD (Taylor, Lees et al. 2002, Trenado, Sudres et al. 2006) and organ transplantation (Golshayan, Jiang et al. 2007, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). In 2008, this TCR transduction approach was adopted by Tsang *et al.* to generate Tregs with indirect allospecificity (Tsang, Tanriver et al. 2008). In this study, Tregs with indirect allospecificity were shown to protect skin and heart allografts significantly more effectively than self-specific Tregs. This approach was expanded on in 2009 when Tregs with dual direct and indirect allospecificity were generated (Joffre, Santolaria et al. 2008, Tsang, Tanriver et al. 2009). These Tregs were able to prolong graft survival more effectively than any population of Tregs found naturally, highlighting the potential for gene-modified cell therapy in the clinic.

1.2.4.2 Regulatory T cell therapy clinical trials

In light of the various studies described above which demonstrated the efficacy of Treg therapy, strategies have been developed over the past decade to adopt Treg therapy in the clinic (Safinia, Leech et al. 2013). Progress was hampered for many years due to difficulties in isolating a sufficient number of Tregs with a reasonable purity, as well as complications in expanding a therapeutically relevant number of cells under good manufacturing practice (GMP) conditions (Bluestone 2005). However, with solutions to these problems being identified, Treg therapy is now a reality in the clinic (Tang and Bluestone 2013).

Although in its infancy, Treg therapy is being investigated in a wide variety of settings ranging from transplantation to various autoimmune diseases, as shown in Table 1-1. The majority of trials in the transplant field employ a similar protocol which is outlined in Figure 1-4. Tregs are isolated from the recipient prior to the transplant procedure, these cells are expanded *ex vivo* and this expanded population of cells is re-infused back into the recipient after the transplant procedure. Further details relating to the isolation and expansion of these cells are discussed in sections 4.1.1 and 4.1.2.

Many factors have been debated to ensure the full potential of the Tregs is exploited, such as the timing, location and dose of administered Tregs. Immediately following the transplant procedure, recipients are typically under a high level of immunosuppression (section 1.1.2.2) which has been shown to dramatically influence the functionality of Tregs in both a positive (Chen, Oppenheim et al. 2006, Lopez, Clarkson et al. 2006, Zeiser, Nguyen et al. 2006, Strauss, Whiteside et al. 2007, Wang, Shi et al. 2009) and negative (Baan, van der Mast et al. 2005, Lopez, Clarkson et al. 2006, Segundo, Ruiz et al. 2006, Zeiser, Nguyen et al. 2006) manner. Combined with the fact that drug use is usually tapered in the months following the transplant procedure, identifying the optimal time to administer the Treg therapy is difficult, although it is clear that a "Treg-supportive" environment is required (Safinia, Leech et al. 2013, Tang and Bluestone 2013). A recent study published by the host laboratory has demonstrated that treating human Tregs *in vitro* with the cocktail of drugs used in The ONE Study compromises the viability of these cells, although their function and stability remains intact (Scotta, Fanelli et al. 2016).

Regarding the location of Treg injection, practical considerations dictate that injection into a peripheral vein is preferable. However, this relies on Tregs expressing the correct homing receptors to ensure they traffic to the graft site or associated lymph nodes. The environment in which the Tregs are expanded dramatically influences the nature of the homing receptors expressed (Scotta, Esposito et al. 2013). Conversely, direct injection into the required site of action (the allograft) may allow the Tregs to exert their protective capacity in a more efficacious manner (Safinia, Leech et al. 2013).

Trials performed to date are Phase I or Phase I/II and as such, are principally investigating the safety of Treg therapy. Consequently, cautious approaches using a limited number of Tregs have been employed thus far. In kidney transplant recipients, polyclonal Treg doses of up to $10x10^6$ Tregs/kg have been demonstrated to be safe in the GMP facility at King's College London. However, in GvHD and T1D contexts, doses of up to $30x10^6$ Tregs/kg have been tolerated (Trzonkowski, Bieniaszewska et al. 2009, Brunstein, Miller et al. 2011, Di Ianni, Falzetti et al. 2011, Marek-Trzonkowska, Mysliwiec et al. 2014, Bluestone, Buckner et al. 2015).

Context	Treg specificity	Trial number	Trial name and location
Kidney transplantation	Polyclonal	NCT02129881	The ONE Study (UK)
Kidney transplantation	Polyclonal	NCT02371434	The ONE Study (Germany)
Kidney transplantation	Polyclonal	NCT02091232	The ONE Study (USA)
Kidney transplantation	Polyclonal	NCT02088931	TASK (USA)
Kidney transplantation	Polyclonal	NCT02145325	TRACT (USA)
Kidney transplantation	Polyclonal	NCT01446484	N/A (Russia)
Kidney transplantation	Direct allospecificity	NCT02711826	TASK (USA)
Kidney transplantation	Direct allospecificity	NCT02244801	The ONE Study DART (USA)
Liver transplantation	Polyclonal	NCT02166177	ThRIL (UK)
Liver transplantation	Direct allospecificity	NCT01624077	N/A (USA)
Liver transplantation	Direct allospecificity	NCT02188719	delta (USA)
Liver transplantation	Direct allospecificity	NCT02474199	ARTEMIS (USA)
GvHD following BMT	Donor-derived	NCT02385019	TREGeneration (Portugal)
GvHD following BMT	Donor-derived	NCT01903473	N/A (Belgium)
GvHD following BMT	Donor-derived	NCT02749084	N/A (Italy)
GvHD following BMT	Donor-derived	NCT01937468	N/A (USA)
GvHD following BMT	Donor-derived	NCT01911039	N/A (USA)
GvHD following BMT	Donor-derived	NCT02526329	N/A (USA)
GvHD following BMT	Donor-derived	NCT01795573	N/A (USA)
GvHD following BMT	Donor-derived	NCT00376519	N/A (USA)
GvHD following BMT	Donor-derived	NCT00725062	N/A (USA)
GvHD following BMT	Cord-derived	NCT00602693	N/A (USA)
GvHD following BMT	Cord-derived	NCT02423915	N/A (USA)
GvHD following BMT	Cord-derived	NCT02118311	N/A (USA)
GvHD following BMT	Cord-derived	NCT01163201	N/A (USA)
T1D	Polyclonal	NCT01210664	N/A (USA)
T1D	Polyclonal	NCT02691247	N/A (USA)
T1D	Polyclonal	NCT02772679	TILT
Lupus erythematosus	Polyclonal	NCT02428309	N/A (USA)
Uveitis	Polyclonal	NCT02494492	UVEREG (France)
Autoimmune hepatitis	Polyclonal	NCT02704338	N/A (USA)

Table 1-1 | **List of clinical trials investigating the safety and efficacy of Treg therapy using tTregs, according to clinicaltrials.gov.** *In cases where Tregs were not isolated from the recipient, this is stated under the "Treg specificity" column. GvHD = graft-versus-host disease, BMT = bone marrow transplantation, T1D = Type 1 diabetes, TASK = Treg adoptive therapy for subclinical inflammation in kidney transplantation, TRACT = Treg adoptive cellular transfer, DART = Donor-alloantigen reactive regulatory T cell, ThRIL =*

therapy of regulatory T cells in liver transplantation, delta = donor-alloantigen-reactive regulatory T cell in liver transplantation, ARTEMIS = Alloantigen reactive Tregs to facilitate minimisation and/or discontinuation of immunosuppression, TILT = T1D immunotherapy using polyclonal Tregs, UVEREG = uveitis regulatory T cell.

Taken together, it is clear that due diligence should be employed regarding the timing, location and dose of Treg therapy. However, another fundamental factor to consider is the specificity of the Tregs. As previously mentioned, current trials expand Tregs in either a polyclonal manner or with allogeneic APCs to yield Tregs with direct allospecificity. With numerous studies supporting the increased efficacy of allospecific Tregs, the latter is preferable. However, this is not the only approach which may be employed to acquire allospecific Tregs. As previously described, Tregs with indirect allospecificity may be generated by transducing polyclonal cells to express specific α and β TCR genes (Joffre, Santolaria et al. 2008, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009). Another approach to generate antigen-specific Tregs is to use chimeric antigen receptors (CAR) (Elinav, Waks et al. 2008, Elinav, Adam et al. 2009, Lee, Hayman et al. 2011, Fransson, Piras et al. 2012, Blat, Zigmond et al. 2014, Jethwa, Adami et al. 2014, MacDonald, Hoeppli et al. 2016). The generation of alloantigen-specific Tregs using CARs is the main topic of this thesis.

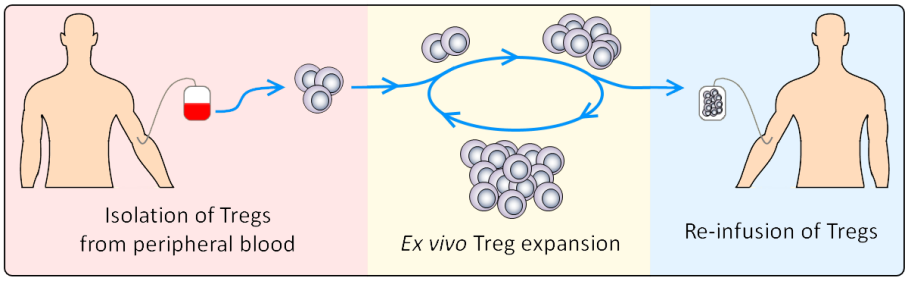

Figure 1-4 | **Schematic diagram outlining the process of Treg therapy.** *To acquire an autologous population of Tregs which may be applied in a Treg therapy protocol, peripheral blood is drawn from the patient and Tregs are isolated either through the use of GMP-compatible fluorescence-assisted cell sorting (FACS) or in vitro negative and positive selection protocols using magnetic beads (red section). The Tregs are then activated, usually in a polyclonal manner using anti-CD3/CD28 beads, and undergo a single or multiple rounds of stimulation in order to expand the number of Tregs ex vivo (yellow section). This expanded population of Tregs is then infused back into the patient, typically as part of a combination therapy protocol (blue section).*

1.3 CHIMERIC ANTIGEN RECEPTORS

The successful generation of antigen-specific T cells is a goal which is sought-after in various fields of research. In a cancer setting, various approaches have demonstrated the therapeutic benefits of adoptive T cell therapy with particular success in the treatment of melanoma (Dudley, Wunderlich et al. 2002, Mackensen, Meidenbauer et al. 2006, Dudley, Yang et al. 2008, Hunder, Wallen et al. 2008). In these trials, tumour infiltrating lymphocytes (TIL) with tumour antigen-specificity are expanded *ex vivo* prior to re-infusion. However, these cells are rare and as such, isolating and expanding clinically applicable number of cells can be complicated.

As described above, one solution that may overcome this shortcoming involves the generation of genetically-engineered antigen-specific T cells. The obvious approach which may be employed is to transduce the T cell with specific α and β TCR chain genes which combine to yield a TCR with a desired specificity. However, early studies which adopted this approach were hampered by the fact that T cells inherently contain their own endogenous TCR genes. Consequently, ¾ of the TCR complexes presented on the surface of these cells were not specific for the desired MHC-peptide complex: ¼ of the TCRs comprised endogenous TCR chains and a further ½ consisted of a mixture of endogenous and transduced α and β chains (Thomas, Xue et al. 2007). It was subsequently demonstrated that these hybrid TCR complexes had the potential to direct the T cell towards self-antigens, promoting the development of autoimmunity (Bendle, Linnemann et al. 2010). Various strategies have been explored to reduce this issue. One such solution is to target T cells with α and β TCR chains which are structurally modified in the constant region, such that they pair with endogenous chains with a low efficiency (Cohen, Li et al. 2007, Kuball, Dossett et al. 2007, Thomas, Xue et al. 2007). Alternatively, strategies to decrease the expression of endogenous α and β chain TCR genes have also been explored using either RNA-interference to knockdown endogenous TCR expression (Ochi, Fujiwara et al. 2011, Provasi, Genovese et al. 2012) or genome editing techniques such as transcription activator-like effector nucleases (TALEN) to knockout endogenous TCR genes (Berdien, Mock et al. 2014).

With an ever-increasing number of solutions to this endogenous/exogenous TCR dimerisation problem coming to light, research into the use of TCR genes to redirect the specificity of T cells prevailed. This approach has had particular success in the generation of melanoma-specific T cells. In 2006, Morgan *et al*. published the results of a T cell therapy-based clinical trial in which autologous T cells were transduced with specific TCR α and β chain genes (Morgan, Dudley et al. 2006). The resulting TCR complex (DMF4) facilitated the recognition of a peptide derived from the melanoma antigen recognised by T cells (MART)-1 antigen (residues 27-35) presented in the context of HLA-A2. TCR transduction increased the proportion of $CD8^+$ T cells presenting the Vβ12

TCR chain from ~1% to 30%. This TCR expression allowed 15% of the transduced T cell to bind a HLA-A*0201 tetramer presenting the MART-1 peptide, compared to ~0.1% of the untransduced T cells. Tumour regression (defined by Response Evaluation Criteria in Solid Tumours (RECIST) criteria (Therasse, Arbuck et al. 2000)) was observed in 2 out of the 15 patients treated. This was improved upon by Johnson *et al.* who used 576 TCR gene sequences from the TILs of three individual melanoma patients to generate a consensus TCR clone (DMF5). This TCR recognised the same MHC-peptide complex as DMF4 but with a higher affinity (Johnson, Heemskerk et al. 2006). When investigated in a similar clinical setting (NCI07C0175), T cells expressing the DMF5 TCR achieved tumour regression (RECIST criteria) in 6 out of 20 patients treated (Johnson, Morgan et al. 2009). Various other successes have been achieved using TCR-transduced T cell therapy for the treatment of various melanomas and sarcomas (Robbins, Morgan et al. 2011, Morgan, Chinnasamy et al. 2013, Morrison 2014).

Although TCR transduction has been successful in the generation of melanoma-specific T cells, this approach is fundamentally flawed as TCRs recognise peptides presented in the context of MHC molecules. MHC molecules are frequently downregulated in tumours and the high

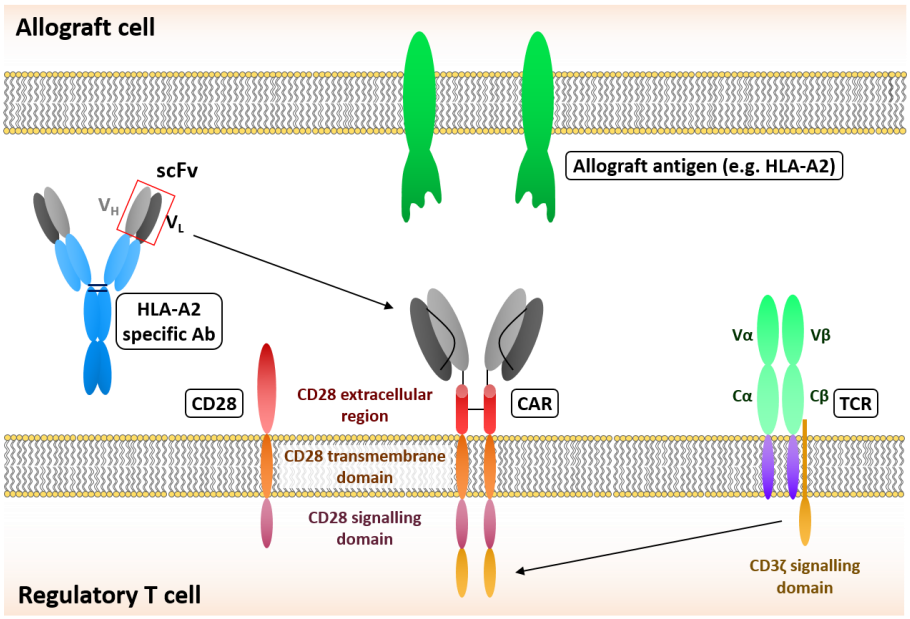

Figure 1-5 | **Schematic diagram detailing the components of a CAR.** *CARs consist of four components: an antigen-specific targeting moiety such as an scFv domain (grey), a hinge domain (red), a transmembrane domain (orange) and an intracellular signalling domain (purple/gold). These various components are obtained from different molecules such as antigen-specific antibodies (blue) which provide the extracellular antigen-binding domain or T cell surface protein complexes and co-stimulatory molecules which make up the intracellular signalling domain. The resulting fusion protein is capable of translating the binding of specific extracellular antigen (green) into intracellular T cell activation signals. Diagram adapted from image provided by Maher (Maher 2012).*

variability of these molecules between patients necessitates the generation of a TCR library for each target antigen presented in the context of each HLA allele, a significant impracticality for translating this to the clinic. This can be circumvented by employing an approach which was first demonstrated by Eshhar *et al.* in 1989 (Gross, Gorochov et al. 1989, Gross, Waks et al. 1989), namely to use CARs to direct the specificity of T cells.

1.3.1 COMPONENTS OF CHIMERIC ANTIGEN RECEPTORS

CARs are synthetic fusion proteins which comprise sequences of various protein complexes to facilitate in the redirection of the specificity of T cells towards desired antigens of interest. Their development has principally been driven by research in a cancer setting which has aimed to generate T cells with tumour antigen specificity. In essence, CARs contain three discrete domains: an extracellular antigen-targeting moiety, a hinge/transmembrane domain and an intracellular signalling entity (Figure 1-5). Together, these domains allow a CAR to translate the extracellular binding of specific antigens into the intracellular activation of desired T cell signalling cascades (Maher 2012).

1.3.1.1 Antigen-targeting moiety

The specificity of a CAR is dictated by the antigen-targeting moiety it incorporates. Although a variety of different macromolecules can be used to dictate the specificity of a CAR (Chmielewski, Hombach et al. 2013), the most common and arguably most robust approach is to use the sequence of an single-chain variable fragment (scFv). Since an scFv is derived from an antibody, this enables CARs to engage target antigens in an MHC-independent manner, a significant advantage over TCRs which recognise peptides presented in the context of MHC molecules.

1.3.1.2 Hinge domain

The antigen-targeting moiety of the CAR is linked to the transmembrane domain via a flexible hinge domain. Over the years, these domains have comprised sequences acquired from many different proteins such as the extracellular region of CD28, CD8, CD4 or the fragment crystallisable (Fc) region of an IgG (Guest, Hawkins et al. 2005, van der Stegen, Hamieh et al. 2015). However, the nature and length of the hinge domain selected can have a dramatic influence on the functionality of a CAR. In 2015, Hudecek *et al*. used a human xenograft tumour

model to demonstrate that T cells expressing a CD19-specific CAR which incorporated a short hinge domain (12 amino acids (aa)) were able to eradicate $CD19^+$ Raji tumours whilst T cells expressing the same CAR with a longer hinge domain (228 aa) could not (Hudecek, Sommermeyer et al. 2015). This response was attributed to the fact that CAR T cells with a long hinge domain underwent AICD. However, Guest *et al*. demonstrated that different length hinges were required, depending on the nature of the target antigen. For epitopes which were physically closer to the cell membrane, a longer, more flexible hinge was required (Guest, Hawkins et al. 2005). Taken together, these studies demonstrated that if a hinge domain was not of a sufficient length, the flexibility of the antigen-targeting moiety was compromised and target antigens were not efficiently engaged. However, if the hinge was too long, the functional efficacy of the T cell was compromised.

1.3.1.3 Transmembrane domain

The transmembrane domain is often considered as a mere structural element which links the extracellular antigen-targeting moiety to the intracellular signalling domain. However, in 2010, Bridgeman *et al*. demonstrated that the nature of this domain can influence how the CAR interacts with other cell surface proteins (Bridgeman, Hawkins et al. 2010). In this study, the authors compared the functionality of various CARs which incorporated the same carcinoembryonic antigen (CEA)-specific targeting moiety but different transmembrane and signalling domains. Through point mutational analysis, it was noted that key residues in the transmembrane domain facilitated dimerisation of the CAR. Similar mutations also abrogated interactions made between the CARs and endogenous members of the TCR/CD3 complex such as the TCRβ chain which was confirmed by Förster resonance energy transfer (FRET). T cells expressing the CARs which contained these mutations were significantly compromised in their ability to respond to immobilised CEA, compared to T cells expressing unmodified CARs. This correlates with findings that T cells require a "threshold" of TCR-mediated signals to be activated (Viola and Lanzavecchia 1996). In this context, it appears that CARs must dimerise and form complexes with endogenous signalling complexes to reach this threshold. As such, the transmembrane domains which are incorporated into CARs are typically derived from proteins which accumulate in the TCR/CD3 complex, namely CD3, CD4, CD8 and CD28.

1.3.1.4 Signalling domain

Since their inception, CARs have evolved through three generation which are defined by the signalling domains incorporated. The first generation CARs, such as those developed by Eshhar *et al*. (Eshhar, Waks et al. 1993) were engineered to mimic endogenous TCRs thus incorporated domains derived from the TCR/CD3 signalling complex such as CD3ζ (Irving and Weiss 1991), providing "signal 1" only (section 1.1.3.2.1). However, despite being functional, these CARs had a low efficacy which was attributed to the lack of a co-stimulatory domain to provide "signal 2" (Heslop 2010, Jena, Dotti et al. 2010, Maher 2012). This was a particular issue in the cancer setting where CARs were originally developed, as tumour cells not only downregulate MHC molecules (Bubenik 2003) but also, do not usually express co-stimulatory molecules (Driessens, Kline et al. 2009). Consequently, second generation CARs were developed to incorporate both a TCR/CD3-derived signalling element to provide "signal 1" and a co-stimulatory molecule-derived element to provide "signal 2" (Maher, Brentjens et al. 2002, Heslop 2010, Maher 2012). The success of these second generation CARs inevitably led to the development of third generation CARs which incorporated multiple co-stimulatory domains from different sources (Maher 2012). Different research groups have developed second and third generation CARs to incorporate signalling domains from a wide variety of co-stimulatory molecules including CD28, 4-1BB, OX40, Lck, ICOS, CD27, 2B4 and DAP10 (Zhao, Wang et al. 2009, Heslop 2010, Jena, Dotti et al. 2010, Maher 2012, van der Stegen, Hamieh et al. 2015).

1.3.2 CHIMERIC ANTIGEN RECEPTORS IN CANCER RESEARCH

Reflecting the application for which they were originally developed, the majority of published CAR studies focus on their use in a cancer context. Indeed, promising results from pre-clinical studies performed using a variety of animal models warranted the adaptation of CAR technology into the clinic (Sadelain, Brentjens et al. 2009). CARs have been assessed clinically for the treatment of a range of cancers with the results of approximately 30 trials published to date (Dai, Wang et al. 2016).

Resounding success has been demonstrated for the treatment of various B cell malignancies using CARs targeting CD19 such as acute (ALL) and chronic lymphocytic leukaemia (CLL) (Kalos, Levine et al. 2011, Porter, Levine et al. 2011, Brentjens, Davila et al. 2013, Grupp, Kalos et al. 2013, Porter, Hwang et al. 2015), non-Hodgkin's lymphoma (Till, Jensen et al. 2012) and B cell lymphoma (Kochenderfer, Wilson et al. 2010, Kohn, Dotti et al. 2011). For example, in the context of ALL, complete remission has been observed in approximately 90% of patients treated in two

clinical trials (27/30 patients by Maude *et al*. (Maude, Frey et al. 2014) and 14/16 patients by Davila *et al*. (Davila, Riviere et al. 2014)) with encouraging results from additional trials consistently being published.

Unfortunately, these successes were not matched when CAR T cells were investigated as a means of destroying solid tumours. Fewer trials have been performed to assess the efficacy of CAR T cells in solid tumour contexts (Dai, Wang et al. 2016) and where trial data has been published, complete remission is rarely observed (Kershaw, Westwood et al. 2006, Park, Digiusto et al. 2007, Pule, Savoldo et al. 2008, Louis, Savoldo et al. 2011, Ahmed, Brawley et al. 2015, Brown, Badie et al. 2015). The most promising results to date were published by Louis *et al*. where complete remission was observed in 3/19 neuroblastoma patients (Louis, Savoldo et al. 2011). These less promising results have attributed to various unique factors of solid tumours such as the lack of infiltrating blood vessels which complicates T cell trafficking into the core of the tumour and once in the tumour, the presence of an immunosuppressive microenvironment.

1.3.3 VARIATIONS ON THE CHIMERIC ANTIGEN RECEPTOR

As a consequence of the modest results which have been achieved with CAR T cells in solid tumour settings, various approaches have been investigated to improve the efficacy of CAR T cells. These approaches typically involve transducing T cells to express multiple exogenous genes. For example, T cells redirected for universal cytokine killing ("TRUCK") are T cells which are engineered to express a CAR and an exogenous cytokine gene (Chmielewski, Hombach et al. 2014, Chmielewski and Abken 2015). In 2011, Chmielewski *et al*. developed TRUCKs which expressed a CEA-specific CAR and secreted high levels of the pro-inflammatory cytokine IL-12 (Chmielewski, Kopecky et al. 2011). The exogenous gene which encoded the IL-12 p35 and p40 subunits was under the control of the $NFAT_6$ minimal promoter and as such, was induced upon CAR engagement (Hooijberg, Bakker et al. 2000). The rationale behind this system was to generate CAR T cells which both elicited an anti-tumour response and attracted innate immune cells such as NK cells and macrophages which could launch a "second wave" immune insult within the tumour (Chmielewski, Hombach et al. 2014). "Armoured CAR" T cells function along similar lines but instead of being restricted to secreting additional cytokines, these cells can instead express ligands which enhance the functionality of the CAR T cell, such as CD40L or 4-1BBL (Pegram, Park et al. 2014, Yeku and Brentjens 2016).

Chimeric cytokine receptor (CCR) are also designed to be used in combination with a CAR. These are cell surface receptors which detect one cytokine but deliver a stimulatory signal which would

naturally come from the detection of a different cytokine. Examples include the IL-4/2 (Wilkie, Burbridge et al. 2010) and IL-4/7 (Leen, Sukumaran et al. 2014) receptors detect extracellular IL-4 using an IL-4 ectodomain, but trigger IL-2 and IL-7 intracellular signalling cascades through the use of IL-2 and IL-7 endodomains, respectively. These receptors can be exploited both *in vitro,* for selectively expanding transduced T cells, and *in vivo,* for promoting the expansion of CAR T cells as IL-4 is present at elevated levels in many tumours (Li, Chen et al. 2009).

Recently, Ellebrecht *et al*. applied CAR technology in a particularly intelligent and elegant manner to generate T cells which targeted autoreactive B cells (Ellebrecht, Bhoj et al. 2016). This study was performed in the context of pemphigus vulgaris, a rare autoimmune blistering disease which is fuelled by autoreactive antibodies which recognise the keratinocyte adhesion protein desmoglein (Dsg)3 (Koga, Tsuruta et al. 2013). To target the B cells which would proceed to secrete this antibody, a chimeric autoantibody receptor (CAAR) was developed which presented Dsg3 extracellularly and contained a CD137-CD3ζ intracellular signalling domain. B cells presenting an antibody specific for Dsg3 engaged the CAAR presented by the T cells and were specifically lysed. This novel approach was shown to effectively eliminate anti-Dsg3 B cells *in vivo* which resulted in the protection of human skin xenografts without off-target toxicity.

1.3.4 Alternative uses for chimeric antigen receptors

CARs have principally been developed to confer antigen specificity onto Tconvs. However, as Tregs are activated in a similar manner to Tconv, it is fathomable that CARs can be used to impose antigen-specificity onto Tregs (Boardman, Maher et al. 2016). This has been achieved in mouse and human cells to treat various autoimmune diseases and prevent undesired immune responses.

In 2008, Elinav *et al.* (Elinav, Waks et al. 2008) generated murine Tregs which expressed a CAR specific for 2,4,6-trinitrophenol (TNP), a colitis-associated model antigen. In this study, Tregs were isolated from transgenic mice expressing the TNP-specific CAR under the control of the CD2 promoter. Upon adoptive transfer, these Tregs prevented the development of 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis, but not oxazolone-induced colitis, highlighting the antigen-specific nature of these Tregs. Furthermore, *in vivo* fluorescence imaging of these cells showed that they accumulated in the colon, suggesting that CAR Tregs trafficked to sites where their cognate antigen is present. These authors also demonstrated that self-specific Tregs retrovirally transduced to express the TNP-specific CAR were equally capable of preventing

colitis (Elinav, Adam et al. 2009) and that similar results could be obtained through targeting different colitis-associated antigens (Blat, Zigmond et al. 2014).

The protective capacity of CAR Tregs has also been demonstrated for the treatment of MS. In 2012, Fransson *et al.* transduced murine $CD4^+$ T cells to express both a CAR specific for myelin oligodendrocyte glycoprotein (MOG) and the *FoxP3* gene, resulting in cells which elicited both a suppressive capacity and functioned in a MOG-dependent manner (Fransson, Piras et al. 2012). These cells were found to localise to the CNS where they alleviated the symptoms of EAE, following adoptive transfer.

The functionality of CARs has also been demonstrated in human Tregs which were developed as a proof of concept (Hombach, Kofler et al. 2009) or as controls for CAR T cells in a cancer study (Lee, Hayman et al. 2011, Jethwa, Adami et al. 2014). However, despite premonitions (Jethwa, Adami et al. 2014), little data was published on CAR Tregs until earlier this year, when MacDonald *et al*. demonstrated the efficacy of human CAR Tregs in a xeno-GvHD model (MacDonald, Hoeppli et al. 2016). In this study, a CAR specific for HLA-A2 was developed, remarkably similar to that which is described in Chapters III and IV. Tregs expressing this CAR were shown to inhibit xeno-GvHD more effectively than polyclonal Tregs in two separate mouse models. In the first model, non-obese diabetic (NOD) severe combined immunodeficiency (SCID) common γ-chain$^{-/-}$ (γ_c; NSG) mice were injected with HLA-$A2^+$ peripheral blood mononuclear cells (PBMC) ± HLA-A2-specific or irrelevant CAR Tregs. In the second model, HLA-A2-transgenic NSG mice were injected with HLA-$A2^-$ PBMCs ± HLA-A2-specific CAR Tregs. Mice in the first model survived significantly longer when HLA-A2-specific CAR Tregs were injected, compared to irrelevant CAR Tregs, whilst in the second model, less damage was observed in the liver and lungs of mice which received HLA-A2-specific CAR Tregs, compared to PBMCs alone.

In addition to these published findings, numerous abstracts pertaining to CAR Tregs have recently been presented at conferences (Naumann, Kim et al. 2014, Skuljec, Chmielewski et al. 2014, Belmonte, Gertner-Dardenne et al. 2016, Fu, Lyle et al. 2016). The biotechnology company "TxCell" has also recently announced the approval of a patent for the development of CAR Tregs for treating various autoimmune and inflammatory diseases (patent identification number: EP 2126054 A2), a pursuit led by Prof Zelig Eshhar (Courme 2016). Furthermore, a partnership between TxCell and "Ospedale San Raffaele" has been announced for the development of CAR Tregs to treat lupus (Courme and Gardini 2016). Taken together, it is evident that an era of research into CAR Tregs is emerging.

1.4 HYPOTHESES AND AIMS

Allospecific Tregs protect allografts significantly more effectively than polyclonal Tregs upon adoptive transfer. However, given the nature of a TCR complex, these $CD4^+$ cells are inherently restricted to recognise peptides presented in the context of MHC class II complexes which are almost exclusively presented by professional APCs. Conversely, donor MHC class I is an alloantigen which is ubiquitously expressed by all donor-derived cells including those which comprise the allograft.

1.4.1 HYPOTHESIS

In a transplant setting, the protection offered by polyclonal Treg therapy could be enhanced by manipulating the Tregs to express a CAR which recognises donor MHC class I in a peptide-independent manner.

1.4.2 AIMS

This project aimed to generate and characterise Tregs which were redirected towards donor MHC class I through the use of CAR technology. This was to be investigated using human Tregs which were redirected towards HLA-A2, and mouse Tregs which were redirected towards K^d. For each CAR, the following aims were set:

- Design and generate a gene encoding a CAR which was specific for the target MHC molecule of interest
- Confirm the functionality and specificity of the CARs using Tconv assays
- Optimise protocols for delivering the CAR genes into target Tregs
    - Confirm this process did not influence fundamental characteristics of these cells
- Compare various functional characteristics of polyclonal and CAR Tregs *in vitro*
- Assess the efficacy with which CAR Tregs protect skin allografts *in vivo*

CHAPTER II

MATERIALS AND METHODS

2.1 SUBJECTS

2.1.1 HUMAN SAMPLES

All human samples were acquired from anonymised healthy donors with informed consent and full ethical approval. Peripheral blood was obtained in the form of leukocyte-enriched blood cones through the National Blood Service (NHS Blood and Transplantation, Tooting, London, UK) with ethical approval from the Institutional Review Board of Guy's Hospital; reference 09/H0707/86. Human umbilical cord endothelial cells (HUVEC) were isolated from umbilical cords provided by the midwifery staff of the Maternity Unit at the Royal London Hospital and prepared using protocols approved by the East London & The City Local Research Committee; reference 05/Q0603/34. Skin explants were obtained from routine abdominoplasty or reduction mammaplasty surgical procedure with ethical approval from Guy's and St. Thomas' NHS Foundation Trust and King's College London; reference 06/Q0704/18.

2.1.2 MICE

A list of the mouse strains used in this thesis is provided in Table 2-1. Mice were delivered to the Biological Services Unit (BSU) of New Hunt's House, King's College London, at least one week prior to any experimental procedure and housed under specific pathogen free (SPF) conditions. All experimental procedures were conducted under sterile conditions in accordance to Home Office regulations; procedure individual licence (PIL) number 70/26014 or I10D8A9CD and procedure project licence (PPL) number 70/7302.

2.2 CELL ISOLATION AND CULTURE

All cell-based procedures and protocols were conducted under sterile conditions in a class II biological safety cabinet using 0.22 µm filtered reagents/solutions. Unless stated otherwise, all cells were cultured in ventilated cell culture flasks or tissue culture treated plates at 37°C in the presence of 5% (v/v) CO_2. All cells were pelleted via centrifugation at a relative centrifugal force (RCF) of 350 g, unless stated otherwise, and counted using a haemocytometer and trypan blue dye (Sigma-Aldrich).

Strain	Description	Breeding/purchase location	Origin
C57BL/6 (B6)	Wild-type mice	Harlan	N/A
BALB/c	Wild-type mice	Harlan	N/A
B6.K^d	B6 mice transgenic for BALB/c MHC class I (K^d)	Charles River	Prof Ralph Patterson Bucy (Department of Pathology, University of Alabama, USA)
OT-II	B6 mice transgenic for TCR α- and β-chain genes which specifically recognised chicken ovalbumin peptide residues 323-339 ($OVA_{323-339}$) presented in the context of B6 MHC class II (I-A^b)	Charles River	Charles River
BALB/c RAG2$^{-/-}\gamma_c^{-/-}$ (BRG)	BALB/c mice with a knockout of the recombination-activating gene 2 (RAG2) and the IL-2 common γ chain (γ_c) gene	Charles River	Prof Adrian Hayday (Division of Immunology and Infectious Diseases, King's College London, UK)
Ch1-2hSa	B6 mice with a knockout of the RAG, γ_c, complement C5, β_2-microglobulin and MHC class II β-chain (I-Aβ^b) genes. These mice were also transgenic for HLA-A2, HLA-DR1 and human signal-regulatory protein (SIRP)α. The SIRPα gene was under the macrophage colony-stimulating factor (CSF)-1 receptor (c-fms) promoter	Charles River	Dr Sylvie Garcia (Department of Immunology, Pasteur Institute, France) (Serra-Hassoun, Bourgine et al. 2014)

Table 2-1 | List of mouse strains used and details on their origins.

2.2.1 CELL CULTURE MEDIA

- **Roswell Park Memorial Institute (RPMI)-1640 human complete cell media** – RPMI-1640, 5% heat-inactivated human serum (HS; Biosera, Nuaille, France), 100 units (U)/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (all from Thermo Fisher Scientific, Paisley, UK).

- **X-vivo complete cell media** – X-vivo 15 (Lonza, Basel, Switzerland) and 5% heat-inactivated HS.

- **Dulbecco's Modified Eagle Medium (DMEM) complete cell media** – DMEM (Lonza) supplemented with 4.5 g/L glucose, 10% heat-inactivated foetal calf serum (FCS; Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine.

- **Iscove's Modified Dulbecco's Medium (IMDM) complete cell media** – Iscove's Modified Dulbecco's Medium (IMDM; Lonza) supplemented with 4.5 g/L glucose and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Thermo Fisher Scientific), 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine.

- **B-LCL media** – RPMI-1640, 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Thermo Fisher Scientific).

- **Standard RPMI cell media** – RPMI-1640, 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine.

- **HUVEC media** – Medium (M)199 (Lonza), 20% heat-inactivated HS (Lonza), 100 U/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich), 2 mM L-glutamine and 2.5 µg/mL Fungizone (Thermo Fisher Scientific)

- **RPMI-1640 mouse complete cell media** – RPMI-1640, 10% heat-inactivated FCS (General Electric (GE) Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES and 50 µM β2-mercaptoethanol (Thermo Fisher Scientific).

2.2.2 HUMAN PBMC ISOLATION AND CULTURE

Human PBMCs were isolated from leukocyte-enriched blood cones by density gradient centrifugation. Cone blood was diluted 8-fold with sterile phosphate buffered saline (PBS; no Ca^{2+} or Mg^{2+}) and carefully layered on top of 15 mL Lymphoprep™ (StemCell™ Technologies, Vancouver, British Columbia, Canada) in a 50 mL Falcon™ tube. The blood was centrifuged at 940 g for 20 minutes at room temperature with an acceleration at 40% of the maximum possible rate and no brake. Buffy coat cells were harvested and washed 3 times with PBS – once at 940 g for 10 minutes followed by 2 washes at 235 g for 10 minutes to remove contaminating platelets.

For experiments in which the isolated PBMCs were to be used for *in vitro* experiments, the cells were suspended at a concentration of $3x10^6$/mL in "RPMI-1640 human complete cell media" (section 2.2.1), activated with 5 µg/mL leucoagglutinin (PHA-L; Sigma-Aldrich, St. Louis, Missouri, USA) and cultured in 6-well plates (4 mL/well) in the presence of 100 U/mL recombinant human IL-2 (Proleukin®, Prometheus Laboratories Inc., San Diego, California, USA).

2.2.2.1 Depletion of $CD25^+$ PBMCs

Total PBMCs were depleted of Tregs using a positive selection protocol for $CD25^+$ cells. PBMCs were suspended in MACS buffer (PBS without Ca^{2+} or Mg^{2+} supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and 5 µM EDTA (Ethylenediaminetetraacetic acid; Thermo Fisher Scientific)) at a concentration of $10x10^6$ cells/mL. Cells were mixed with CD25 MicroBeads II (Miltenyi Biotec, Cologne, Germany) at a 4:1 v/v ratio and incubated for 15 minutes at 4°C under rotation. The cells were then washed once with MACS buffer in a volume of 50 mL to remove excess beads and then suspended in 3 mL MACS buffer.

An LS column with accompanying pre-separation filter (Miltenyi Biotec) was inserted into a MACS separator magnet and rinsed with 3 mL MACS buffer. The PBMC suspension prepared above was added to the column and the effluent containing unlabelled $CD25^-$ PBMCs was collected. The column was washed 3 times with 3 mL MACS buffer to collect as many $CD25^-$ PBMCs as possible and the column containing the $CD25^+$ PBMCs was discarded. The $CD25^-$ PBMCs were then cryopreserved in freezing medium (90% heat-inactivated FCS and 10% Dimethyl sulfoxide (DMSO)) and stored in liquid nitrogen until use.

2.2.3 Human $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ Tconv isolation and culture

Human $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Tconvs were isolated concurrently from leukocyte-enriched blood cones using a negative selection protocol for $CD4^+$ T cell followed by a positive selection protocol for $CD25^+$ cells. Cone blood was diluted 2-fold with PBS (without Ca^{2+} or Mg^{2+}) and incubated with RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail (150 µL RosetteSep™ per 5 ml blood; StemCell™ Technologies) for 30 minutes at room temperature. This process crosslinked the unwanted $CD4^-$ cells (expressing CD8, CD16, CD19, CD36, CD56, CD66b or TCRγ/δ) with the red blood cells (RBC). The blood was then diluted an additional 4-fold with PBS, layered on top of 15 mL Lymphoprep™ and centrifuged as detailed above (section 2.2.2). Buffy coat ($CD4^+$) cells were harvested and CD25 MicroBeads II were used to separate the $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Tconvs, as detailed above (section 2.2.2). $CD4^+CD25^+$ Tregs were eluted by removing the column from the magnet, adding 5 mL MACS buffer to the column and using a low pressure generated with a plunger. The purity of the $CD4^+CD25^+$ Tregs was assessed by flow cytometry immediately following isolation using an anti-human CD4 APC antibody (OKT4 clone) and an anti-human CD25 PE antibody (2A3 clone) and was consistently found to be >90% (Figure 4-1A). Alternatively, an extensive phenotype was conducted using the antibodies detailed in Table 2-4 (Figure 4-1B).

In order to establish a Treg cell line, freshly-isolated cells were suspended in "X-vivo complete cell media" (section 2.2.1) at a concentration of $1x10^6$ cells/mL and activated with anti-human

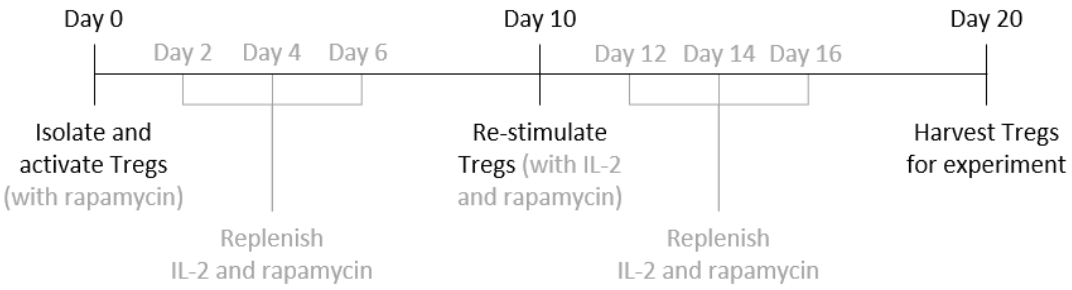

Figure 2-1 | **Timeline showing when human Tregs were re-stimulated and used for experiments, relative to the time of isolation.** *Human Tregs were activated with anti-human CD3/CD28 beads and cultured in the presence of 100 nM rapamycin on the day of isolation (day 0). On day 2, cultures were supplemented with fresh rapamycin and 1,000 U/mL recombinant human IL-2. IL-2 and rapamycin was replenished on days 4 and 6 of culture. On day 10, the Tregs were harvested, washed and re-cultured in fresh media with IL-2, rapamycin and anti-human CD3/CD28 beads. IL-2 and rapamycin was replenished on days 12, 14 and 16 and the cells were used for experimentation on day 20.*

CD3/CD28 Dynabeads® Human T-Activator beads (Thermo Fisher Scientific) at a 1:1 cell:bead ratio. The Tregs were cultured in 24-well plates with $1x10^6$ cells/well in the presence of 100 nM rapamycin (LC-Laboratories, Woburn, Massachusetts, USA). On days 2, 4 and 6 post-stimulation, cultures were supplemented with 1,000 U/mL human recombinant IL-2 and rapamycin was replenished. A timeline for the Treg expansion protocol is provided in Figure 2-1.

On day 10 of culture, resting Tregs were harvested and the anti-CD3/CD28 beads were removed using a magnet. The cells were washed with PBS and suspended in "X-vivo complete cell media" supplemented with IL-2 and rapamycin. The cells were then re-stimulated with anti-human CD3/CD28 beads at a 1:1 cell:bead ratio and cultured as detailed above. Tregs were starved of IL-2 and harvested before being used in experiments on day 20 (Figure 2-1).

2.2.4 IMMORTALISED ADHERENT CELL LINE CULTURE

Human Embryonic Kidney (HEK) 293T cells were kindly donated by Dr Gilbert Fruhwirth (Division of Imaging Sciences and Biomedical Engineering, King's College London, UK). H29D, PG13, MCF-7 and T-47D were all kindly provided by Dr John Maher (Division of Cancer Studies, King's College London, UK). Phoenix-Ecotropic (Eco) cells were kindly donated by Dr Angelika Holler and Prof Hans Stauss (Institute of Immunity and Transplantation, University College London, UK).

HEK293T cells are a derivative of HEK293 cells (Graham, Smiley et al. 1977) which have been modified to stably express the Simian Vacuolating Virus 40 (SV40) large T-antigen. The presence of this SV40 T-antigen allows HEK293Ts to efficiently replicate vectors which contain the SV40 origin of replication (DuBridge, Tang et al. 1987).

H29D cells are a derivative of HEK293 cells which have been modified to express the Moloney murine leukaemia virus (MoMuLV) *gag-pol* polyprotein (Ory, Neugeboren et al. 1996). Furthermore, these cells have been engineered to express the vesicular stomatitis virus-G (VSV-G) envelope (*env*) protein under the control of a tetracycline-regulatable gene expression system (Gossen and Bujard 1992), as constitutive expression of VSV-G is toxic in HEK293 cells. Consequently, the VSV-G protein is only expressed when these cells are grown in the absence of tetracycline (Suzuki and Suzuki 2011).

PG13 cells are derived from NIH/3T3-thymidine kinase $(TK)^-$ mouse fibroblasts (Wei, Gibson et al. 1981). Like H29D cells, these cells have been modified to express the MoMuLV *gag-pol* polyprotein but instead of producing VSV-G-pseudotyped retroviral particles, PG13 cells produce

Gibbon ape leukaemia virus (GaLV)-pseudotyped particles (Miller, Garcia et al. 1991, Gallardo, Tan et al. 1997).

MCF-7 and T-47D cells are well-established human breast cancer epithelial cell lines. MCF-7 is an adenocarcinoma cell line which was first isolated in 1970 from a 69-year-old patient (Soule, Vazguez et al. 1973) whilst T-47D is a carcinoma cell line which was first isolated in 1979 from a 54-year-old patient (Keydar, Chen et al. 1979).

Phoenix-Eco cells are a derivative of HEK293T cells which have been modified by Nolan G. *et al.* (Stanford University, California, USA) to express a *gag-pol* polyprotein in addition to an ecotropic *env* protein. As such, retroviral particles produced by this cell line are capable of infecting mouse and rat cells only.

HEK293T, PG13, MCF-7 and T-47D cells were all maintained in "DMEM complete cell media" (section 2.2.1). H29D cultures were supplemented with 2 µg/mL tetracycline which was replenished every two days, unless expression of the *env* glycoprotein VSV-G was desired (section 2.4.1), in which case the cells were grown in the absence of tetracycline. Phoenix-Eco cells were maintained in "IMDM complete cell media" (section 2.2.1). To harvest the cells, all cell culture media was removed and the cells were washed once with PBS before being incubated with Trypsin-EDTA (0.05%; Thermo Fisher Scientific) for 2-3 minutes. The cells were then washed with their respective culture media and pelleted before being re-seeded at a desired concentration in cell culture flasks. Freshly thawed cells were grown for at least 1 week before use in experiments. Cells were passaged when they reached a confluency of 80-90% and were expanded for a maximum of 20 passages.

2.2.5 HUVEC ISOLATION AND CULTURE

HUVECs were isolated and cultured by Dr Dianne Cooper (William Harvey Research Institute, Queen Mary University of London, UK).

Umbilical cords were transported and stored in cord buffer (Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific) containing 100 U/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich) and 2.5 µg/mL Fungizone) at 4°C until processing. HUVECs were isolated from the interior umbilical vein by collagenase digestion (Jaffe, Nachman et al. 1973). Residual blood was washed from the cord using approximately 30 mL PBS and a butterfly needle. The cord was clamped at both ends, filled with approximately 20-25 mL pre-warmed collagenase solution (serum-free medium M199 containing 0.1% collagenase type II (Sigma-Aldrich), 100 U/mL

penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 2.5 µg/mL Fungizone) and incubated at 37°C for 15 minutes. The collagenase solution was collected and the cord was rinsed with 30 mL PBS. Wash out solution was also collected. Cells were pelleted at 300 g for 10 minutes and suspended in HUVEC media (section 2.2.1).

HUVECs were cultured in T75 flasks pre-treated with 0.5% bovine gelatin. All cells isolated from each cord (1-2x10^6 cells/cord) were incubated in the T75 flask. The cell culture media was changed after 24 hours and every 48 hours from this point onwards. Cells were split 1:3 when a confluency of 90-95% was reached using 0.5% Trypsin-EDTA (Thermo Fisher Scientific), as detailed in section 2.2.4. HUVECs were used in experiments at passage 2-3.

2.2.6 B-LCL CELL CULTURE

Four B-LCLs were purchased from the International Histocompatibility Working Group (IHWG, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA): DBB (IHW09052; HLA-A2$^+$DR7$^+$), MOU (IHW09050; HLA-A2$^-$DR7$^+$), SPO (IGW09036; HLA-A2$^+$DR11$^+$) and BM21 (IGW09043; HLA-A2$^-$DR11$^+$). These cells were grown in "B-LCL media" (section 2.2.1). Freshly thawed cells were grown for at least 1 week before use in experiments and were expanded for a maximum of 20 passages.

2.2.7 K562 CELL CULTURE

Three K562 cell lines were kindly donated by Dr Marc Martinez-Llordella (Institute of Liver Sciences, King's College London, UK): empty K562s and K562s stably transfected with either HLA-A2 or HLA-A1. These cells were cultured in "standard RPMI cell media" (section 2.2.1) which was supplemented with 5 µg/mL puromycin (Thermo Fisher Scientific) to selectively expand HLA-transfected cells, where appropriate. Freshly thawed cells were grown for at least 1 week before use in experiments and were expanded for a maximum of 20 passages.

2.2.8 HYBRIDOMA CELL CULTURE AND ANTIBODY PRODUCTION

A BALB/c MHC class I (K^d)-specific hybridoma cell line (HB-159™, clone SF1-1.1.10) was purchased from the American Type Culture Collection (ATCC®, Manassas, Virginia, USA). These cells were cultured in "standard RPMI cell media" (section 2.2.1), as per supplier's protocols. K^d-specific antibody was purified from these cells using Two-Compartment Bioreactor cell culture flasks

(CELLine CD350, Integra Biosciences, Zizers, Switzerland), as per manufacturer's protocols. Briefly, 5-8x10^6 cells were suspended in 5 mL Protein-Free Hybridoma Media (PFHM-II; Thermo Fisher Scientific) and carefully added to the 'cell compartment' of the Two-Compartment Bioreactor cell culture flask whilst the 'medium compartment' was filled with 250 mL "standard RPMI cell media" with 5% FCS (section 2.2.1). After 1 week of culture, the cells were harvested from the cell compartment, pelleted and the supernatant containing anti-K^d antibody was collected and stored at 4°C. 5-8x10^6 cells were then suspended in 5 mL PFHM-II and re-cultured in the cell compartment with a fresh 250 mL media in the medium compartment. This process was repeated twice a week until 15 mL supernatant containing anti-K^d antibody was collected.

Anti-K^d antibody was concentrated using a two-step filtration approach. Supernatant containing the antibody was filtered using a 0.22 µm filter. Contaminants of <30 kDa were removed using a Centriprep® 30K Centrifugal Filter Device (Merck Millipore, Darmstadt, Germany). These devices utilise a membrane filter which allows molecules of ≤30 kDa to pass through, separating them from molecules of >30 kDa. Devices were washed with 15 mL PBS prior to use. 15 mL of the supernatant was added to the 'sample container' of the device, after which the 'filtrate collector' was added. Devices were centrifuged at 1,500 g for 20 minutes at 4°C and supernatant remaining in the 'sample container' (depleted of molecules ≤30 kDa) was collected. This supernatant was further concentrated using an Amicon® Ultra-15 100K Centrifugal Filter Device (Merck Millipore). These devices use a similar membrane-based approach to retain molecules ≥100 kDa. As before, the device was washed with 15 mL PBS before use. Supernatant was added to the 'filter device' of the implement which was centrifuged at 4,000 g for 30 minutes at 4°C. Concentrated supernatant remaining in the 'filter device' was collected and stored at 4°C for future use.

Concentrated anti-K^d antibody was quantified using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). 25 µL samples of the antibody (neat, 1:10 and 1:100 diluted) were added to wells of a 96-well U-bottom plate alongside a 2-fold serial dilution of BSA (ranging from 2 mg/mL to 16 µg/mL) which was used to generate a standard curve. All conditions were performed with 2 technical replicates. A 'working reagent' (WR) was prepared by mixing BCA Reagent A with BCA Reagent B in a 50:1 (v/v) ratio. 200 µL of this WR was added to each sample in the 96-well plate and the plate was incubated at 37° for 30 minutes. Absorbance was measured at 540 nm and the protein concentration was calculated using the standard curve.

2.2.9 MURINE BM-DC ISOLATION AND CULTURE

DC progenitors were isolated from B6 mice using a negative selection protocol. BM from the tibia, femur and pelvic bones was extracted and passed through a 70 µm nylon cell strainer to produce a single cell suspension. RBCs were lysed by incubating the cells in ammonium chloride potassium (ACK) buffer (H_2O containing 40.3 mM NK_4Cl, 10.0 mM $KHCO_3$ and 0.127 mM EDTA) at room temperature for 1.5 minutes after which the cells were washed with RPMI-1640. Leukocytes were incubated with rat anti-mouse CD8 (YTS-169), CD4 (YTS-191), MHC class II (M5-114) and B220 (RA3-3A1) antibodies (all made in house from hybridomas) for 30 minutes at 4°C, under rotation. Leukocytes were then washed to remove excess antibodies and incubated with pre-washed magnetic beads coated with polyclonal anti-rat IgGs (Dynal® beads, Thermo Fisher Scientific) for a further 30 minutes at 4°C, under rotation. Dynal® beads binding contaminating cells were removed using a magnet, according to the manufacturer's protocol. DC progenitors were washed with RPMI-1640 and cultured in "RPMI-1640 mouse complete cell media" (section 2.2.1) supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF; 20 ng/mL; made in house) in 24-well plates ($1x10^6$ cells/mL/well). Media was changed on days 2 and 4 post-isolation. Day 6 BM-DCs were irradiated (30 Gray (Gy)) and used for mouse Treg stimulation.

2.2.10 MOUSE TREG CULTURE

A B6 self-specific Treg cell line which was previously generated by Tsang *et al*. was used in this thesis (Tsang, Tanriver et al. 2008). This cell line was established by using $CD4^+CD25^+$ T cells which were enriched from the secondary lymphoid organs of B6 mice using the Dynabeads® FlowComp™ Mouse $CD4^+CD25^+$ Treg Cells Kit (Thermo Fisher Scientific), as per manufacturer's protocols. These cells were then stimulated once a week with irradiated syngeneic BM-DCs (section 2.2.9).

To re-stimulate the cells, the Tregs were harvested, washed twice with RPMI-1640 and suspended at $2x10^6$ cells/mL in "RPMI-1640 mouse complete cell media" (section 2.2.1). Day 6 immature BM-DCs were irradiated (30 Gy), washed with RPMI-1640 and suspended at $1x10^6$ cells/ml in "RPMI-1640 mouse complete cell media" (section 2.2.1). The Tregs and irradiated DCs were cultured together at a 1:4 ratio (1 mL of Tregs with 0.5 mL of irradiated DCs) in 24-well plates. 10 U/mL recombinant human IL-2 was added on days 1, 2 and 4 of culture and day 7 Tregs were used for experiments.

2.3 MOLECULAR BIOLOGY

2.3.1 PCR AMPLIFICATION OF TARGET GENES

Custom polymerase chain reaction (PCR) primers (Table 3-1) were synthesised by Sigma-Aldrich and reconstituted at a concentration of 50 µM in distilled (d)H_2O. PCR reactions were set up to consist of 10 ng template DNA (SFG_A2-28ζ (Table 2-3)), 0.25 µM of a forward and reverse primer, 25 µL Phusion® High-Fidelity DNA Polymerase (New England BioLabs® Inc., Ipswich, Massachusetts, USA) and DNase-free H_2O to a final volume of 50 µL. Reactions were then run on the following PCR program to yield the desired PCR product:

- 98°C for 3 minutes
- 35 cycles of:
  - 98°C for 30 seconds
  - A ramp cooling at a rate of 0.5°C/second from 98°C to 72°C
  - 72°C for 50 seconds
- 72°C for 5 minutes
- Hold at 4°C

2.3.2 RESTRICTION DIGESTION

Restriction digestions were performed in a total volume of 10-50 µL with a maximum final DNA concentration of 40 ng/µL using enzymes provided by New England Biolabs® Inc., according to manufacturer's protocols. Target DNA was diluted with DNase-free H_2O and mixed with a buffer appropriate for the enzyme being used (Table 2-2). Where NEBuffer 3.1 was used, digestion mixtures were supplemented with 100 µg/mL BSA. Respective restriction enzymes were then added to the reaction mixtures with a minimum final concentration of 0.2 U/µL. Reaction mixtures were incubated at a respective temperature suitable for the restriction enzyme being used (Table 2-2) for a minimum of 1 hour which was adjusted accordingly when >1 µg DNA was being digested (1 hour per µg DNA). For double enzyme digests, the most suitable buffer was identified using the "NEB Double Digest Finder". If 2 enzymes had different working temperatures, sequential digests were performed starting at the lower temperature. Where appropriate, re-ligation of digested plasmids was inhibited by adding alkaline phosphatase (AP; New England Biolabs® Inc.) at a final concentration of 0.4 U/µL into the digestion mixture and incubating for 45 minutes at 37°C.

Enzyme	Buffer	Temperature	Company
BamHI	NEBuffer 3.1	37°C	New England BioLabs® Inc.
BstBI	CutSmart® Buffer	60°C	New England BioLabs® Inc.
ClaI	CutSmart® Buffer	37°C	New England BioLabs® Inc.
MluI	NEBuffer 3.1	37°C	New England BioLabs® Inc.
NcoI	NEBuffer 3.1	37°C	New England BioLabs® Inc.
NotI	NEBuffer 3.1	37°C	New England BioLabs® Inc.
PvuII	CutSmart® Buffer	37°C	New England BioLabs® Inc.
XhoI	NEBuffer 3.1	37°C	New England BioLabs® Inc.

Table 2-2 | List of restriction enzymes and the respective conditions in which they were used.

Digestion products were separated on a 0.8% agarose gel prepared by mixing 0.8 g agarose (Sigma-Aldrich) with 100 mL Tris-acetate-EDTA (TAE) buffer (Severn Biotech Ltd., Kidderminster, Worcestershire, UK). Digest products were mixed with a 6X loading buffer (5:1 DNA:loading buffer v/v ratio; New England BioLabs® Inc.) and loaded into the gel. Gels were run with limits of 130 mA and 80 V until a sufficient separation was achieved.

2.3.3 DNA PURIFICATION FROM PCR AMPLIFICATION PRODUCTS AND AGAROSE GEL

DNA was extracted and purified from PCR amplifications and agarose gel bands using the Wizard® SV Gel and PCR Clean-Up System (Promega), as per manufacturer's protocols. DNA fragments from restriction digestions (section 2.3.2) were separated using a 0.6% agarose gel and fragments containing DNA of interest were excised. 1 µL Membrane Binding Solution was added per mg of DNA/agarose gel and the mixture was incubated at 60°C until the agarose gel dissolved. Alternatively, Membrane Binding Solution was added to PCR amplification products (section 2.3.1) at a 1:1 (v/v) ratio.

Dissolved agarose gel or PCR product mixtures were added to SV Minicolumns and incubated for 1 minute at room temperature before being centrifuged at 16,000 g for 1 minute. Minicolumns containing the DNA of interest were washed twice by adding 500-700 µL Membrane Wash Solution and centrifuging at 16,000 g for 1 minute. A final 1 minute centrifuge at 16,000 g was performed in the absence of Membrane Wash Solution to ensure all residual buffer was removed. The DNA was then eluted into 30 µL Nuclease-Free Water by centrifugation at 16,000 g for

1 minute. Eluted DNA was re-centrifuged through the same SV Minicolumn to maximise DNA recovery.

2.3.4 PLASMID LIGATION

The quantity of DNA from restriction digested inserts and target vectors was estimated by running specific volumes of the DNA on a 0.8% agarose gel and comparing the band intensities to Hyperladder™ I (Bioline Reagents, London, UK) bands. Ligation reactions were performed using a total of 40-100 ng DNA with insert:vector molar ratios ranging from 1:1 to 3:1.

To blunt-end ligate inserts into the pCR™ vector, the ZeroBlunt® PCR Cloning Kit (Thermo Fisher Scientific) was used, as per manufacturer's protocols. Insert and vector DNA was mixed with 1X ExpressLink™ T4 DNA Ligase Buffer and DNase-free H_2O to a final volume of 9 µL. 1 µL (5 U) of the ExpresLink™ T4 DNA Ligase was then added to the mixture before being incubated at room temperature for 30 minutes.

For ligation reactions in which sticky ends were available, insert and vector DNA was mixed in DNase-free H_2O to a final volume of 17 µL. This DNA was mixed with 2 µL 10X T4 DNA Ligase Reaction Buffer (New England BioLabs® Inc.). T4 DNA Ligase (New England BioLabs® Inc.) was then added to each reaction at a final concentration of 20 U/µL which was then run on the following ligation program in a PCR machine:

- 160 cycles of:
  - 16°C for 3 minutes
  - 10°C for 30 seconds
  - 24°C for 30 seconds
- 16°C for 5 hours
- 65°C for 15 minutes
- Hold at 4°C

2.3.5 BACTERIAL TRANSFORMATION

Constructs were replicated by transforming either MAX Efficiency® DH5α™ competent or One Shot® Stbl3™ chemically competent *Escherichia coli* cells (both from Thermo Fisher Scientific). Cells were thawed on ice and 100 ng DNA in a volume of <2.5 µL was added to a vial containing 25 µL cells. The DNA and cells were mixed gently and incubated on ice for 30 minutes. The cells

were then heat-shocked for 45 seconds at 42°C and placed on ice for a further 2 minutes. 250 µL pre-warmed super optimal broth with catabolite repression (SOC) media (37°C) was added to each vial which was subsequently incubated under shaking conditions for 1 hour at 37°C. 80 µL of the transformed cells were then spread on pre-warmed Lysogeny broth (LB)-agar (Sigma-Aldrich) plates supplemented with an antibiotic appropriate for the construct of interest (100 µg/mL ampicillin (Sigma-Aldrich) or 50 µg/mL kanamycin (Sigma-Aldrich)). Plates were incubated at 37°C overnight to yield bacterial colonies which were picked using a sterile pipette tip and expanded as detailed in section 2.3.6.

2.3.6 MINIPREPARATION

Minipreps were performed using the Promega Wizard® Plus SV Minipreps DNA Purification System, according to manufacturer's protocols. Bacterial colonies, prepared as detailed in section 2.3.5, were picked and inoculated in LB broth (Sigma-Aldrich) supplemented with an appropriate antibiotic (100 µg/mL ampicillin or 50 µg/mL kanamycin). Cells were initially grown in a volume of 2 mL for 6-8 hours at 37°C under shaking conditions. The culture media volume was then increased to 10 mL and the bacteria were incubated at 37°C overnight.

Glycerol stocks of each bacterial culture were prepared by mixing 100 µL of an overnight culture with 50 µL sterile solution of 50% glycerol (diluted with Milli-Q H_2O; (Sigma-Aldrich)) and stored at –80°C.

Bacterial cultures were centrifuged at 1,780 g for 10 minutes at 4°C. Pelleted bacteria were suspended in 250 µL Cell Resuspension Solution and transferred to sterile 1.5 mL microcentrifuge tubes. An equal volume of Cell Lysis Solution and 10 µL Alkaline Protease Solution were added and mixed by inversion. Cell lysis reactions were incubated at room temperature for 5 minutes and neutralised by adding 350 µL Neutralization Solution. Cleared lysates, which were obtained by centrifuging each sample at 18,400 g for 10 minutes at room temperature, were transferred to Spin Columns within Collection Tubes and centrifuged at 18,400 g for 1 minute to facilitate binding of plasmid DNA to the column. Columns containing DNA were washed twice by adding 750 µL Wash Buffer and centrifuging at 18,400 g for 1 minute. A final centrifuge at 18,400 g for 1 minute was performed to remove residual buffer. The spin columns were then transferred to fresh microcentrifuge tubes, 50 µL DNase-free H_2O (pre-warmed to 60°C) was added to each column and the columns were centrifuged at 18,400 g for 1 minute to elute the DNA. DNA was quantified by nanodrop and stored at –20°C for future use.

2.3.7 MAXIPREPARATION

Constructs obtained by miniprep (section 2.3.6) were validated by restriction digest analysis (section 2.3.2) and sequence analysis. To acquire large preparations of desired constructs, 20 µL of the corresponding glycerol stock, prepared as detailed in section 2.3.6, was inoculated into 200 mL LB broth supplemented with an appropriate antibiotic. Bacterial cultures were then incubated under shaking conditions at 37°C overnight.

Maxipreps were performed using the PureLink® HiPure Plasmid Filter Maxiprep Kit (Thermo Fisher Scientific), as per manufacturer's protocols. The density of transformed *E. coli* present in the overnight cultures was assessed by photospectrometry, measuring the absorbance at 600 nm. Absorbance values of cultures diluted 1:10 were typically between 0.2 and 0.4 OD_{600} units. For each maxiprep, the equivalent of 300 OD_{600} units of bacteria was used.

PureLink® HiPure Maxi Columns were equilibrated by adding 30 mL Equilibration Buffer directly to the Filtration Cartridge within each column and allowing the solution to elute by gravity. The desired quantity of bacteria was pelleted by centrifugation at 1,780 g for 10 minutes at 4°C. Pelleted bacteria were suspended in 10 mL Resuspension Buffer supplemented with RNaseA by vortex/pipetting until the solution was homogenous. Bacteria were lysed by adding 10 mL Lysis Buffer to each cell suspension, mixing by inversion and incubating at room temperature for 5 minutes. The lysis reaction was neutralised by adding 10 mL Precipitation Buffer to each solution. Cell lysates were added to the equilibrated columns and allowed to drain by gravity. Once the solution had eluted, the Filtration Cartridge within the column was discarded and the column containing the DNA was washed with 50 mL Wash Buffer. The DNA was eluted by adding 15 mL Elution Buffer to the column and allowing the solution to drain by gravity.

The 15 mL eluted DNA was mixed with 10.5 mL isopropanol (7:3 DNA:isopropanol v/v ratio; Sigma-Aldrich) and centrifuged at 4,025 g for 1 hour at 4°C. Supernatants were discarded and the pelleted DNA was washed by suspending in 5 mL 70% ethanol (diluted with Milli-Q H_2O; Sigma-Aldrich) and centrifuged again at 4,025 g for 5 minutes at 4°C. Supernatants were discarded and DNA pellets were air-dried before being suspended in 400 µL Tris-EDTA (TE) buffer. DNA was quantified by nanodrop and stored at –20°C for future use.

2.4 VIRUS PRODUCTION AND CELL TRANSDUCTION

2.4.1 RETROVIRAL PARTICLE PRODUCTION FOR TRANSDUCING HUMAN CELLS

To generate a stable retroviral packaging cell line, a two-step protocol was employed, as outlined by Parente-Pereira A *et al.* (Parente-Pereira, Wilkie et al. 2014). H29D cells were transfected with the SFG retroviral vector containing the A2 CAR gene (Table 2-3) using a Calcium Phosphate Transfection Kit (Sigma-Aldrich), as per manufacturer's protocols.

H29D cells (section 2.2.4) were seeded in 6-well plates and transfected when at a confluency of 80-90%. The culture media of these cells was replaced with 3 mL fresh "DMEM complete cell media" (section 2.2.1) without tetracycline 2-3 hours prior to transfection. A solution containing of 2.5 M $CaCl_2$, 5 µg SFG_A2-28ζ DNA and DNase-free H_2O in a final volume of 60 µL was prepared and incubated at room temperature for 5 minutes before being added dropwise to an equal volume (60 µL) of 2X HeBS. This mixture was incubated at room temperature for 20 minutes to allow for precipitation before being added dropwise to the H29D cells. Media of the H29D cells was replaced with fresh "DMEM complete cell media" (section 2.2.1) without tetracycline 24 hours post-transfection. Viral supernatants from the H29D cells were harvested 3, 4, 5 and 6 days post-transfection and used to transduce PG13 cells.

Empty PG13 cells (section 2.2.4) were transduced in 6-well plates when at a confluency of 20-30%. This was achieved by replacing the PG13 cell media with supernatant from the transfected H29D cells containing VSV-G-pseudotyped retroviral particles. PG13 cells were then incubated for 72 hours to allow for transduction and generation of a stable retroviral packaging cell line. PG13 cells which were transduced using H29D supernatant taken 5 days post-transfection were transduced with the highest efficiency (data not shown) thus these PG13 cells were maintained in culture and used for downstream experiments. These cells were maintained in "DMEM complete cell media" (section 2.2.1) and supernatants from these cells was used to transduce target cells, as detailed in section 2.4.2.

2.4.2 RETROVIRAL TRANSDUCTION OF HUMAN CELLS

Stably transduced PG13 cells (section 2.4.1) consistently generated viable retroviral particles containing the SFG vector of interest. In preparation for transducing human T cells, PG13 cells were seeded in 6-well plates and expanded until they reached a confluency of 80-90%. The cell

Construct	Construct description	Principle open reading frame description	Protein name
SFG_NK1-28ζ	RV expression vector	CAR specific for NK1 with human CD28-CD3ζ signalling domain	NK1 CAR
SFG_A2-28ζ	RV expression vector	CAR specific for HLA-A2 with human CD28-CD3ζ signalling domain	A2 CAR
SFG_V8	RV expression vector	Non-specific CAR with human CD29-CD3ζ signalling domain	V8 CAR
SFG_P4	RV expression vector	CAR specific for PSMA with human CD28-CD3ζ signalling domain	P4 CAR
pLNT/SFFV_eGFP	LV expression vector	Cytoplasmic eGFP	eGFP
pLNT/SFFV_A2-28ζ-eGFP	LV expression vector	HLA-A2-specific CAR with human CD28-CD3ζ signalling domain and eGFP fusion	A2 CAR-eGFP
pLNT/SFFV_A2-eGFP	LV expression vector	HLA-A2-specific CAR with eGFP fusion (no signalling domain)	A2 ΔCAR-eGFP
pLNT/SFFV_A2-28ζ	LV expression vector	HLA-A2-specific CAR with human CD28-CD3ζ signalling domain (no eGFP fusion)	A2 CAR
pLNT/SFFV_A2	LV expression vector	HLA-A2-specific CAR (no signalling domain or eGFP fusion)	A2 ΔCAR
pCMV-ΔR8.91	LV packaging plasmid	HIV-I g*ag-pol* polyprotein	N/A
pCMV-VSV-G	LV envelope plasmid	VSV-G envelope protein	N/A
pMP71-PRE_K^d-28ζ-eGFP	RV expression vector	K^d-specific CAR with mouse CD28-CD3ζ signalling domain and eGFP fusion	K^d CAR-eGFP
pMP71-PRE_K^d-eGFP	RV expression vector	K^d-specific CAR with eGFP fusion (no signalling domain)	K^d ΔCAR-eGFP
pCL-Eco	RV packaging plasmid	MoMuLV gag-*pol-env* polyprotein	N/A

Table 2-3 | **Summary of the constructs used, a description of the open reading frames (ORF) within these constructs and the designated name given to the proteins coded by these ORFs.** *RV = retroviral, LV = lentiviral, eGFP = enhanced green fluorescent protein, MoMuLV = Moloney murine leukaemia virus.*

culture media of these cells was then replaced with 2 mL fresh "DMEM compete cell media" (section 2.2.1) 24 hours prior to transduction.

RetroNectin®-coated plates were prepared by incubating non-tissue culture treated plates with 50 µg/mL RetroNectin® diluted in PBS (Clontech/TaKaRa Bio Inc., Kusatsu, Shiga Prefecture, Japan) overnight at 4°C. 24-well and 6-well plates were coated with 300 µL and 2 mL RetroNectin®, respectively. RetroNectin® was removed and the wells washed twice with PBS prior to use.

Unfractioned T cells, selectively activated using PHA-L from PBMCs, were transduced 72 hours post-activation (section 2.2.2) in 6-well RetroNectin®-coated plates. T cells were harvested, washed with PBS and suspended at a concentration of $0.5x10^6$ cells/mL in "RPMI-1640 human complete media" (section 2.2.1). $1x10^6$ T cells (2 mL) were plated into RetroNectin®-coated wells of 6-well plates and mixed with an equal volume of PG13-derived viral supernatant. To aid transduction, the cells were then spinoculated at 600 g for 1 hour at room temperature before being incubated at 37°C. IL-2 was replenished every 2 days until the time of experiment.

T cells were used 7-10 days post-transduction at which point the transduction efficiency was assessed by flow cytometry. As the CARs expressed in these cells lacked a fluorescent reporter (Figure 3-2B), the transduction efficiency was assessed by staining the cells with a fluorescently-conjugated antibody specific for the cMyc 9E10 epitope (EQKLISEEDL; Figure 3-2B), as detailed in section 2.5.1.

2.4.3 LENTIVIRAL PARTICLE PRODUCTION AND CONCENTRATION

Lentiviral particles were generated using a 2nd generation packaging system approach. $5.25x10^6$ HEK293T cells (section 2.2.4) were seeded per T75 flask 18 hours prior to transfection. 24 µg DNA consisting of the plasmid of interest (pLNT/SFFV_A2-28ζ-eGFP or pLNT/SFFV_A2-eGFP), a packaging plasmid (cytomegalovirus p(CMV)-ΔR8.91) and an envelope plasmid (pCMV-VSV-G) were mixed at a 4:3:1 ratio (w/w/w) in 1.5 mL DMEM supplemented with 2 mM L-glutamine only. Polyethylenimine (PEI; Sigma-Aldrich) was added to the DNA/DMEM mixture at a PEI:DNA ratio of 3:1 (w/w) and the mixture was incubated at room temperature for 15 minutes. The media of the HEK293T cells was then replaced with the DNA/DMEM/PEI mixture and fresh "DMEM complete cell media" (section 2.2.1) in a final volume of 8 mL and the cells were incubated at 37°C for 48-56 hours.

Transfection of the HEK293T cells was confirmed visually by fluorescence microscopy 48-56 hours post-transfection. Viral particles were harvested and concentrated 10-fold using polyethylene glycol (PEG)-it™ Virus Precipitation Solution (System Biosciences, Mountain View, California, USA), according to manufacturer's protocols. Briefly, HEK293T cell supernatant containing the viral particles was harvested and filtered through a 0.45 µm filter to remove cell debris before being mixed with cold PEG-it™ solution (4:1 supernatant:PEG-it™ v/v ratio) and incubated at 4°C for 18 hours. The viral particles were then pelleted by centrifugation at 1,500 g for 30 minutes at 4°C before being re-suspended in a desired cell media and frozen at –80°C for later use.

2.4.4 LENTIVIRAL TRANSDUCTION

Target human Tregs or Tconvs were transduced in 24-well RetroNectin®-coated plates (section 2.4.2) 72 hours post-activation, unless stated otherwise. Cells were transduced in the same media they were originally cultured, in the presence of IL-2 but absence of rapamycin. Concentrated viral particles were added to the RetroNectin®-coated wells and mixed with $5x10^5$ target cells. The final concentration of viral particles was 4-fold that of neat viral supernatant, unless stated otherwise. Tregs or Tconvs were then spinoculated at 600 g for 1 hour at room temperature before being incubated at 37°C. The cells were given fresh media supplemented with IL-2 and rapamycin, where appropriate, 18 hours and 72 hours post-transduction (days 4 and 6 post-activation; a detailed timeline for IL-2 and rapamycin replenishment provided in Figure 2-1).

2.4.5 RETROVIRAL PARTICLE PRODUCTION FOR TRANSDUCING MOUSE CELLS

For transducing mouse T cells and Tregs, retroviral particles were produced using a protocol optimised and kindly donated by Dr Angelika Holler and Prof Hans Stauss (Institute of Immunity and Transplantation, University College London, UK). $1.5x10^6$ Phoenix-Eco cells (section 2.2.4) were seeded in a 10 cm cell culture dish, 24 hours prior to transfection, and the culture media of these cells was replaced with 5 mL fresh "IMDM complete cell media" (section 2.2.1) 30-60 minutes before transfection.

The Phoenix-Eco cells were co-transfected with the pMP71_K^d-28ζ-eGFP or pMP71_K^d-eGFP construct and the pCL-Eco retroviral packaging vector using FuGENE® HD (Promega). The original pMP71-PRE constructs were provided by Dr Angelika Holler and Prof Hans Stauss with the express

permission of Prof Christopher Baum and Prof Axel Schambach (Hannover Medical School, Hannover, Germany). 2 solutions were prepared in isolation: 'solution A' which contained 10 µL FuGENE® HD and 150 µL Opti-MEM® Reduced Serum Medium (Thermo Fisher Scientific) and 'solution B' which contained 2.6 µg pMP71_K^d-28ζ-eGFP or pMP71_K^d-eGFP and 1.5 µg pCL-Eco in a total volume of 50 µL. 'Solution A' and 'solution B' were gently mixed and incubated for 15-20 minutes at room temperature before being added dropwise to the Phoenix-Eco cells.

After 24 hours, the culture media of the transfected Phoenix-Eco cells was removed, the cells were gently washed with PBS and the media was replaced with pre-warmed "RPMI-1640 mouse complete cell media" (section 2.2.1). The transfected Phoenix-Eco cells were then incubated for a further 24 hours, after which the "RPMI-1640 mouse complete cell media" containing retroviral particles was harvested. This viral supernatant was centrifuged at 300 g for 5 minutes to remove contaminating cells and cell debris before use.

2.4.6 Retroviral transduction of mouse cells

Murine cells were activated using different approaches, depending on the cell type being transduced. For T cell transductions, spleens were explanted and total splenocytes were liberated by passing through a 70 µm nylon cell strainer using "RPMI-1640 mouse complete cell media" (section 2.2.1). RBCs in the splenocytes were lysed using ACK buffer, as detailed in section 2.2.9. The splenocytes were then activated with 2 µg/mL Concanavalin A (ConA; Sigma-Aldrich) and cultured in "RPMI-1640 mouse complete cell media" (section 2.2.1) supplemented and 1 ng/mL recombinant human IL-7 (R&D Systems) at a concentration of $1.5x10^6$ cells/mL in 24-well plates (2 mL/well). Activated splenocytes were transduced 24 hours post-activation. In contrast, Tregs from a previously established cell line (Tsang, Tanriver et al. 2008) were transduced 48 hours following stimulation with irradiated BM-DCs (section 2.2.10).

All murine cells were transduced in 24-well RetroNectin®-coated plates (section 2.4.2) which were blocked with 2% BSA for 30 minutes at room temperature and washed twice with PBS prior to use. Cells were harvested, washed with RPMI-1640 and suspended in Phoenix-Eco cell-derived supernatant containing retroviral particles (section 2.4.5) at a concentration of $4x10^6$ cells/mL (supplemented with 10 U/mL recombinant human IL-2 for Treg transductions). $2x10^6$ cells (0.5 mL) were added to each well of a 24-well RetroNectin®-coated plate and the cells were spinoculated at 450 g for 1.5 hours at room temperature before being incubated at 37°C.

Transduced splenocytes were given fresh media supplemented with 10 U/mL recombinant human IL-2, 24 hours post-transduction (day 2 post-activation) and harvested for analysis and

downstream applications 72 hours post-transduction (day 4). Alternatively, transduced Tregs were given fresh media supplemented with 10 U/mL recombinant human IL-2, 24 hours (day 3) and 48 hours (day 4) post-transduction and were harvested for analysis and downstream applications 5 days post-transduction (day 7).

2.5 FLOW CYTOMETRY

2.5.1 FLOW CYTOMETRIC ANALYSES

All cells were stained in fluorescence assisted cell sorting (FACS) buffer (PBS without Ca^{2+} or Mg^{2+}, 5 mM EDTA and 1% heat inactivated FCS). Analyses involving human cells were performed using $5x10^5$ cells/sample whilst analyses of mouse cells were performed using $2x10^5$ cells/sample. For standard phenotype analysis, cells were stained in FACS tubes in a total volume of 100 µL. In co-culture assays, cells were stained in 50 µL and used for analysis.

Dead cells were labelled with the LIVE/DEAD® Fixable Near-infra red (IR) Dead Cell Stain Kit, as per manufacturer's protocols, prior to antibody staining. Cells were harvested and washed twice with PBS to remove contaminating FCS before being suspended in 0.5 mL PBS containing a 1:1,000 dilution of the LIVE/DEAD® stock solution (reconstituted in DMSO) and incubated for 30 minutes at 4°C. Cells were then washed twice with 2 mL FACS buffer prior to antibody staining.

For mouse cells only, non-specific antibody binding was blocked by incubating the cells with 5 µg/mL anti-CD16/CD32 (clone 93) for 20 minutes at 4°C. For human cells, no blocking step was performed. The cells were then incubated with fluorescently-conjugated antibodies specific for antigens of interest (Table 2-4 and Table 2-5) for 20 minutes at 4°C. For antibodies which were supplied with stock concentration values, cells were stained with 2-5 µg/mL. Alternatively, manufacturer-specified antibody volumes were used. Following staining, cells were washed twice with 2 mL FACS buffer.

For intracellular staining, cells were fixed and permeabilised with Foxp3/Transcription Factor Staining Buffer (eBioscience, Santa Clara, California, USA), according to manufacturer's protocols. Cells were incubated in 0.5 mL fixation/permeabilisation for 1 hour at 4°C and washed twice with 2 mL permeabilisation buffer. Cells were then stained in 100 µL permeabilisation buffer with desired fluorescently-conjugated antibodies for 30 minutes at 4°C and washed twice with 2 mL FACS buffer.

Specificity	Flourochrome	Clone	Company
CD3	APC-H7	SK7 (Leu-4)	BD Biosciences
CD4	PerCP Cy5.5	OKT4	eBioscience
CD25	Brilliant Violet 605™	2A3	BD Biosciences
CD25	PE	4E3	eBioscience
CD127	PE	A019D5	BioLegend
FOXP3	eFluor® 660	PCH101	eBioscience
CTLA-4	Brilliant Violet 421™	BNI3	BD Biosciences
CD39	PE-Cy7	eBioA1	eBioscience
CCR9	PerCP Cy5.5	L053E8	BioLegend
CCR4	Brilliant Violet 605™	L291H4	BioLegend
CCR10	PE	6588-5	BioLegend
Integrin β7	APC	FIB504	BioLegend
CD62L	Brilliant Violet 421™	DREG-56	BioLegend
CLA	PE-Cy7	HECA-452	BioLegend
CD69	PE-Cy7	H1.2F3	eBioscience
HLA-A2	PE-Cy7	BB7.2	eBioscience
HLA-A2/A28	PE-Vio770	REA142	Miltenyi Biotec
HLA class I	APC	Tu149	Thermo Fisher Scientific
HLA-DR	Alexa Fluor®647	L243	BioLegend
cMyc	FITC	9E10	Sigma-Aldrich
cMyc	APC	9E10	R&D Systems

Table 2-4 | **Summary of the fluorescently-conjugated antibodies used for staining human markers.**

Following the final wash, all cells were suspended in 400 µL FACS buffer for acquisition. Data was acquired on either a BD FACSCalibur™ or BD LSRFortessa™ (BD Biosciences, Franklin Lakes, New Jersey, USA) and analysed using FlowJo 7 (for calculating division indices only) or FlowJo 10 software (Tree Star, Ashland, Oregon, USA).

2.5.2 FLUORESCENCE-ASSISTED CELL SORTING

Transduced cells were harvested, washed twice with 50 mL FACS buffer and suspended at a maximum concentration of $20x10^6$ cells/mL. Desired $eGFP^+$ cells were purified using a BD

Specificity	Flourochrome	Clone	Company
CD4	PE-Cy7	GK1.5	eBioscience
CD25	PE-Cy7	PC61	BD Biosciences
FoxP3	APC	FJK-16s	eBioscience
CTLA-4	APC	UC10-4B9	eBioscience
CD39	PE	24DMS1	eBioscience
CD73	PE	eBioTY/11.8	eBioscience
CD11c	PerCP-Cy5.5	N418	eBioscience
CD80	Brilliant Violet 421™	16-10A1	BioLegend
CD86	Brilliant Violet 605™	GL-1	BioLegend
MHC I (K^d)	PE	SF1-1.1.1	eBioscience
MHC class I (K^b)	APC	AF6-88.5.5.3	eBioscience
MHC class II ($I\text{-}A^d$)	FITC	39-10-8	BioLegend
MHC class II ($I\text{-}A^b$)	FITC	AF6-120.1	eBioscience
CD40	PE-Cy7	3/23	BioLegend
cMyc	APC	9E10	R&D Systems

Table 2-5 | Summary of the fluorescently-conjugated antibodies used for staining mouse markers.

FACSAria II (BD Biosciences). Human cells were sorted into X-vivo complete cell media (section 2.2.1) and mouse cells were sorted into 100% FCS.

2.6 *IN VITRO* ASSAYS

2.6.1 TREG SUPPRESSION ASSAYS

The suppressive capacity of human and mouse Tregs was measured *in vitro* by assessing the efficacy with which these cells inhibited $CD4^+$ responder T cell (Tresp) proliferation. Tresps were activated in either a polyclonal or antigen-dependent manner and co-cultured with the Tregs in 96-well U-bottom plates, as detailed below.

2.6.1.1 Human polyclonal suppression assay

The suppressive capacity of human Tregs was assessed after 20 days of expansion (Figure 2-1). $CD4^+CD25^-$ Tresps autologous to the Tregs being analysed (section 2.2.3) were thawed in PBS and washed 3 times to remove contaminating DMSO and FCS. The Tresps were then labelled with 2 µM CellTrace Violet (CTV; Thermo Fisher Scientific), according to manufacturer's protocols. Briefly, $CD4^+CD25^-$ Tresps were suspended at a concentration of $20x10^6$ cells/mL in pre-warmed PBS only. A solution of pre-warmed PBS containing 4 µM CTV was prepared and added to the Tresps at a 1:1 (v/v) ratio. The cells were incubated for 15 minutes at 37°C after which the CTV labelling reaction was quenched by adding an equal volume of cold FCS and incubating the cells on ice for 5 minutes. Labelled Tresps were washed twice with "X-vivo complete cell media" (section 2.2.1) before being suspended at a concentration of $1x10^6$ cells/mL in "X-vivo complete cell media" and incubated on ice until required.

Human Tregs were harvested and anti-CD3/CD28 beads removed using a magnet. The cells were washed twice with PBS and suspended at a concentration of $1x10^6$ cells/mL in "X-vivo complete cell media". Immediately prior to co-culture, fresh anti-human CD3/CD28 beads were added to the Tresps at a 1:40 bead:cell ratio. $1x10^5$ Tresps (100 µL) with anti-CD3/CD28 beads were plated into a 96-well U-bottom plate with a varying number of Tregs to achieve a desired Tresp:Treg ratio in a final volume of 200 µL.

Co-cultures were incubated at 37°C for 5 days after which Tresp proliferation was measured by flow cytometry. Co-cultures were pelleted and supernatants were harvested and frozen for cytokine analysis. The cells were washed twice with PBS and dead cells were labelled as detailed in section 2.5.1. Cells were then washed twice with FACS buffer and data was acquired by flow cytometry (section 2.5.1). Data was analysed with FlowJo 7 software using division indices. A summary of the gating strategy is provided in Figure 2-2.

2.6.1.2 Human antigen-specific suppression assay

Human Treg antigen-specific suppression assays were performed using a similar approach to the polyclonal suppression assay protocol outlined above (section 2.6.1.1). Tregs and autologous Tresps were prepared as previously detailed. However, Tresp were not activated with anti-human CD3/CD28 beads but instead with B-LCLs described in section 2.2.6. B-LCLs were harvested, washed twice with PBS and suspended at a concentration of $1x10^6$ cells/mL in "X-vivo complete cell media" (section 2.2.1).

Tregs and Tresps (without anti-CD3/CD28 beads) were plated, as previously detailed, into a 96-well U-bottom plate with $3x10^4$ B-LCL (30 µL; 3:1 Tresp:B-LCL ratio) in a final volume of 230 µL. Co-cultures were incubated for 5 days after which Tresp proliferation was analysed, as previously detailed (section 2.6.1.1).

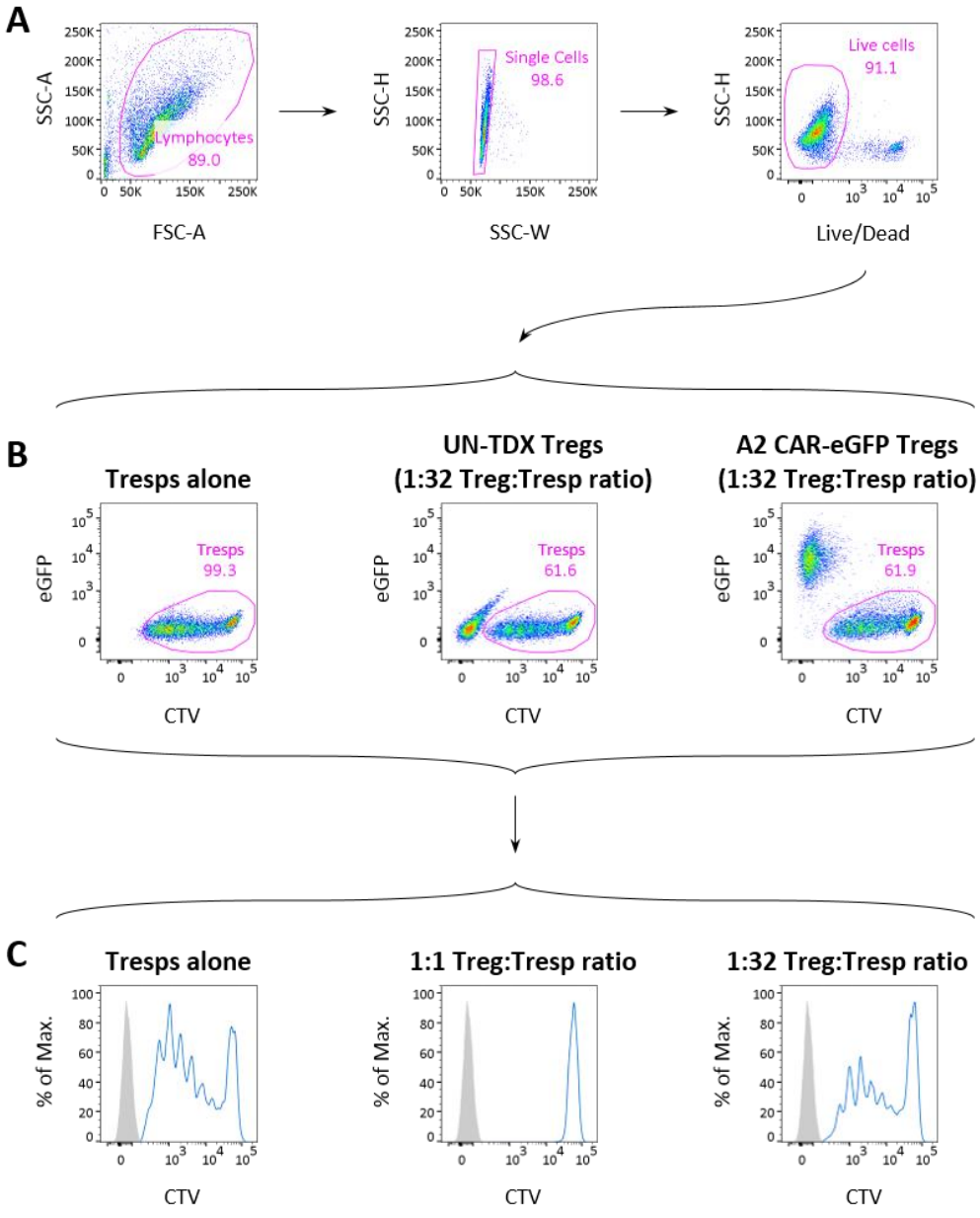

Figure 2-2 | **Gating strategy for suppression assay analysis.** *Representative data shown is taken from a polyclonal human Treg suppression assay in which human Tregs (untransduced or lentivirally transduced to express the A2 CAR-eGFP construct) are co-cultured with $1x10^5$ autologous CellTrace Violet (CTV)-labelled $CD4^+CD25^-$ responder T cells (Tresp) in the presence of anti-human CD3/CD28 beads (1:40 bead:Tresp ratio). A: Total cells were gated after which doublets and dead cells were excluded. B: Live Tresps were gated based on the presence of CTV and lack of eGFP, where appropriate. C: Gated Tresps were analysed for the extent to which they proliferated using division indices in FlowJo 7 software.*

2.6.1.3 Mouse suppression assay

Mouse suppression assays were performed by co-culturing Tregs with freshly-isolated APCs (section 2.6.1.3.1) and Tresps. For polyclonal suppression assays, B6 Tregs were co-cultured with $5x10^4$ B6 $CD4^+$ T cells and $1x10^5$ B6 APCs in the presence of 1 µg/mL anti-mouse CD3ε (145-2C11 clone; BD Pharmingen™, BD Biosciences). For antigen-specific suppression assays, Tregs were co-cultured with Tresps and APCs isolated from specific strains of mice, depending on the nature of the experiment being performed, in the absence of anti-mouse CD3ε. For linked suppression assays, chicken ovalbumin peptide ($OVA_{323-339}$; Thermo Fisher Scientific) was added to co-cultures at a final concentration of 0.2 µg/mL.

2.6.1.3.1 *APC isolation*

Mouse splenocytes depleted of RBCs were acquired as detailed in section 2.4.6 and incubated with rat anti-mouse CD8 (YTS-169) and CD4 (YTS-191) antibodies (made in house from hybridomas) for 30 minutes at 4°C, under rotation. Splenocytes were then washed to remove excess antibodies and incubated with pre-washed magnetic beads coated with polyclonal anti-rat IgGs (Dynal® beads, Thermo Fisher Scientific) for a further 30 minutes at 4°C, under rotation. Dynal® beads binding contaminating T cells were removed using a magnet, according to manufacturer's protocol. Remaining APCs were washed with RPMI-1640, suspended at a concentration of $2x10^6$ cells/mL in "RPMI-1640 mouse complete cell media" and incubated on ice until required. A phenotype analysis of these APCs, performed by flow cytometry, demonstrated that >90% of these APCs expressed MHC class II (Figure 2-3A).

2.6.1.3.2 *$CD4^+$ Tresp isolation*

$CD4^+$ T cells were isolated from mouse splenocytes (section 2.4.6) using a Dynabeads® Untouched™ Mouse $CD4^+$ Cells Kit (Thermo Fisher Scientific). Briefly, RBC-free splenocytes were suspended at $1x10^8$ leukocytes/mL in Isolation Buffer, labelled with a CD4 negative selection 'Antibody Mix' and contaminating $CD4^-$ T cells were removed using a magnet. $CD4^+$-enriched splenocytes were suspended at a concentration of $1x10^6$ cells/mL in "RPMI-1640 mouse complete cell media" (section 2.2.1) and incubated on ice until required. The purity of these $CD4^+$ Tresps was >90% (Figure 2-3B), as confirmed by flow cytometry.

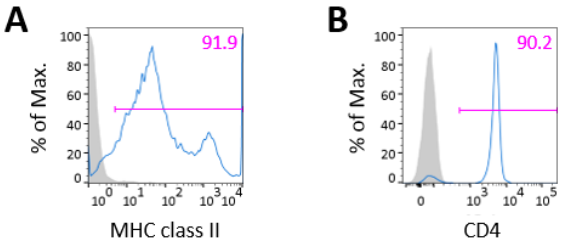

Figure 2-3 | **Purity of APCs and CD4 T cells isolated from mouse spleens.** *A: Proportion of BALB/c splenocytes which expressed I-A^d immediately following an APC-enrichment protocol. Splenocytes were depleted of CD4$^+$ and CD8$^+$ T cells by negative selection. Remaining cells were analysed for expression of I-A^d by flow cytometry. B: Purity of CD4$^+$ T cells following enrichment. Splenocytes were enriched for CD4$^+$ T cells by negative selection. The purity of this protocol was assessed by flow cytometry. Stained cells are shown with a blue line with the percentage of cells expressing the antigen of interest (according to the gate provided) is provided in the top right corner of each plot. Isotype control stains are shown in solid grey.*

2.6.1.3.3 *Murine suppression assay setup*

Tregs were harvested and suspended at 1x10^6 cells/mL in "RPMI-1640 mouse complete cell media" (section 2.2.1). Co-cultures consisting of 5x10^4 CD4$^+$ T cells, 1x10^5 APCs and 5x10^4 Tregs were plated out by adding 50 µL of each respective cell suspension to appropriate wells of a 96-well U-bottom plate. A final volume of 200 µL/well was achieved by adding "RPMI-1640 mouse complete cell media", with or without anti-mouse CD3ε. Co-cultures were incubated at 37 °C for 72 hours, after which Tresp proliferation was assessed.

CD4$^+$ Tresp proliferation was assessed either by CTV dilution or ^{3}H-thymidine incorporation. For CTV-based assays, Tresps were labelled with CTV prior to co-culture and proliferation was measured after 3 days using flow cytometry, as detailed in section 2.6.1.1. For ^{3}H-thymidine-based assays, co-cultures were pulsed with 1 µCi ^{3}H-thymidine/well (Amersham GE Healthcare, UK) for the last 18 hours of culture. Cell proliferation was then measured using a beta-plate scintillation counter (LKB Wallac 1205 Betaplate® liquid scintillation counter, Austria). Data is presented as counts per minute (cpm), representing cells in which ^{3}H-thymidine has been incorporated upon division.

2.6.2 Human Treg activation and proliferation assays

Human Treg activation and proliferation assays were performed by co-culturing Tregs with irradiated (120 Gy) K562s (section 2.2.7) or B-LCLs (section 2.2.6) as artificial APCs. Tregs and APCs

were prepared as detailed in sections 2.6.1.1 and 2.6.1.2, respectively. For activation assays, $1x10^5$ Tregs were co-cultured with $2.5x10^4$ APCs in a 96-well U-bottom plate. CD69 expression was then measured after 18 hours of co-culture by flow cytometry (section 2.5.1). For proliferation assays, $5x10^4$ Tregs were co-cultured with $5x10^4$ APCs in "X-vivo complete cell media" (section 2.2.1) which was supplemented with 10 U/mL IL-2 in 96-well U-bottom plates. Proliferation was measured by ^{3}H-thymidine incorporation after 72 hours, as detailed in section 2.6.1.3.3.

2.6.3 Mouse T cell proliferation assays

Murine proliferation assays were performed by co-culturing transduced splenocytes with freshly-isolated APCs. Splenocytes were harvested 3 days post-transduction (section 2.4.6) and APCs were prepared as described in section 2.6.1.3.1. $1x10^5$ splenocytes were co-cultured with varying numbers of APCs in a final volume of 200 µL "RPMI-1640 mouse complete cell media" in 96-well U-bottom plates. Proliferation was assessed either by ^{3}H-thymidine incorporation or CTV-dilution (section 2.6.1.3.3) thus where appropriate, splenocytes were labelled with 2 µM CTV (section 2.6.1.1) prior to co-culture. Proliferation was measured after 72 hours, as previously described.

2.6.4 Co-culture of human T cells and breast cancer cell monolayers

The specificity and functionality of the human CARs was assessed by investigating the ability of human CAR T cells to kill target cell monolayers expressing the antigen-target of interest, upon co-culture. Human PBMCs or $CD4^+CD25^-$ T cells were isolated, activated and transduced as detailed in sections 2.2 and 2.4, respectively. T cells were harvested 7 days post-transduction and suspended at a concentration of $1x10^6$ cells/mL in their respective cell culture media. $1x10^6$ T cells/well were then co-cultured with 90% confluent MCF-7 and T-47D breast cancer epithelial cell monolayers (section 2.2.4) in a 24-well plate at 37°C overnight. Following co-culture, the viability of the cell monolayers and cytokine production was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and enzyme-linked immunosorbent assay (ELISA), respectively, as detailed below.

2.6.4.1 MTT assay

Supernatants were harvested and cell monolayers were gently washed twice with PBS to remove contaminating T cells. Remaining monolayer cells were cultured in 500 µL/well "DMEM cell culture media" (section 2.2.1) containing 500 µg/mL MTT for 2 hours at 37°C. Viable cells were quantified based on their mitochondrial activity which reduced the soluble yellow dye MTT into the insoluble purple formazan. After 2 hours, all culture media was removed and 300 µL DMSO was added to each well to dissolve the purple formazan. Absorbance was measured at 560 nm. Data is presented as "% viability", relative to the OD_{560} of cell monolayers cultured alone.

2.6.4.2 IL-2/IFNγ/IL-10 ELISA

IL-2, IFNγ and IL-10 ELISAs were performed using Ready-SET-Go!® ELISA kits (eBioscience), as per manufacturer's protocols. NUNC MaxiSorp® 96-well plates (VWR) were coated with 1X Coating Buffer containing a cytokine-specific 'capture antibody' overnight at 4°C. Plates were washed for 1 minute under shaking conditions 3 times with Wash Buffer (PBS containing 0.05% (v/v) Tween-20) using an ELISA plate washer (Thermo Scientific) before being blocked by incubating with 1X ELISA/ELISPOT Diluent at room temperature for 1 hour. Plates were washed 3 times as above and incubated with cell culture supernatants overnight at 4°C. A 2-fold serial dilution standard curve containing 9 points was included in each plate and assays were performed with 3 technical replicates per sample. Where necessary, sample supernatants were diluted up to 50-fold with 1X ELISA/ELISPOT Diluent to ensure the final OD_{450} remained within the range of the standard curve. Plates were washed 5 times as above and incubated with a cytokine-specific biotin-conjugated 'detection antibody' for 1 hour at room temperature. Plates were washed a further 5 times as above and incubated with Avidin-Horse Radish Peroxidase (HRP) for 30 minutes at room temperature. Plates were then washed 7 times (2 minutes/wash) before being incubated with 1X tetramethylbenzidine (TMB) substrate solution for 20 minutes at room temperature. Reactions were stopped by adding 1 M H_3PO_4, absorbance was measured at 450 nm and cytokine quantities were calculated using the standard curve.

2.6.5 Flow chamber assay of human Tregs across HUVEC monolayers

Experiments involving HUVECs were performed by Dr Dianne Cooper at the William Harvey Research Institute (Queen Mary University of London, UK).

HUVECs were cultured in HUVEC media (section 2.2.1) supplemented with 15 ng/mL IFNγ (R&D Systems) for 72 hours prior to experimentation to upregulate expression of HLA molecules. HUVECs were seeded in µ-Slides VI 0.4 (Ibidi, Munich, Germany) pre-coated with 0.5% bovine gelatin such that they were confluent 24 hours later at the time of experimentation.

Tregs were harvested, suspended at $1x10^7$ cells/mL in Dulbecco's PBS (DPBS) without Ca^{2+} or Mg^{2+} containing 0.1% BSA and stored on ice until required. Immediately before flowing, the Tregs were diluted to $1x10^6$ cells/mL in DPBS with Ca^{2+} or Mg^{2+}. Tregs were flowed using a shear stress of 1 dyn/cm^2 generated with an automated syringe pump (Harvard Apparatus Ltd, Harvard Bioscience Inc., Holliston, Massachusetts, USA). Tregs were flowed for 8 minutes prior to recording 10 second frames from 6 random fields of view per condition, observed using a Retiga EXi digital video camera (Q-Imaging, Surrey, British Columbia, Canada) and recorded in StreamPix capture software (NorPix Inc., Montreal, Quebec, Canada). Sequences were loaded into ImagePro-Plus software (Media Cybernetics, Rockville, Maryland, USA), Tregs were manually tagged and their migration monitored. 3 measurements were made in the analysis: the total number of interacting cells which initially captured during the 10 second frame, those which were firmly adherent (remain stationary for the 10 second observation period) and cells which transmigrated (migrated through the endothelial monolayer).

2.7 *IN VIVO* ASSAYS

2.7.1 XENO-GVHD

Immunodeficient Ch1-2hSa mice, 6-7 weeks of age, were injected intravenously (iv) with $1x10^7$ $CD25^-$ PBMCs ± $2x10^6$ autologous Tregs isolated from a $HLA\text{-}A2^-$ donor. Following cell transfer, the mice were monitored every 2-3 days for signs of xeno-GvHD including, but not limited to, weight loss, hunching, reduced mobility, ruffled hair or orbital inflammation. End-points were defined prior to experimentation but were not reached.

2.7.2 HUMAN SKIN XENOGRAFT TRANSPLANTATION

Human skin grafts were transplanted by Dr Lesley Smyth.

Human skin was obtained from routine surgical procedures, stored in sterile PBS and grafted onto mice within 12 hours of initial explantation. Skin was disinfected using PBS containing 100 U/mL

penicillin and 100 µg/mL streptomycin and split-thickness skin explants were prepared using a dermatome. Skin explants were cut into 1.5 cm^2 grafts using a scalpel, kept moist using sterile gauze swabs saturated with PBS containing 100 U/mL penicillin and 100 µg/mL streptomycin and stored at 4°C until required.

Skin allografts were transplanted onto recipient BRG mice, 10-11 weeks of age, as previously described (Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). Mice were administered 2 µg Vetergesic Multidose analgesia (Alstoe Ltd., Sogeval, Laval, France) by subcutaneous injection and put under general anaesthesia using Isoflurane (Merial, Sandringham, UK). Skin on the back of the mice was shaved and disinfected using Vetasept povidone-iodine antiseptic solution (Animalcare Ltd., York, UK). A square of mouse skin measuring approximately 1.5 cm^2 was explanted and replaced with the human skin allograft. The allograft was held in place using

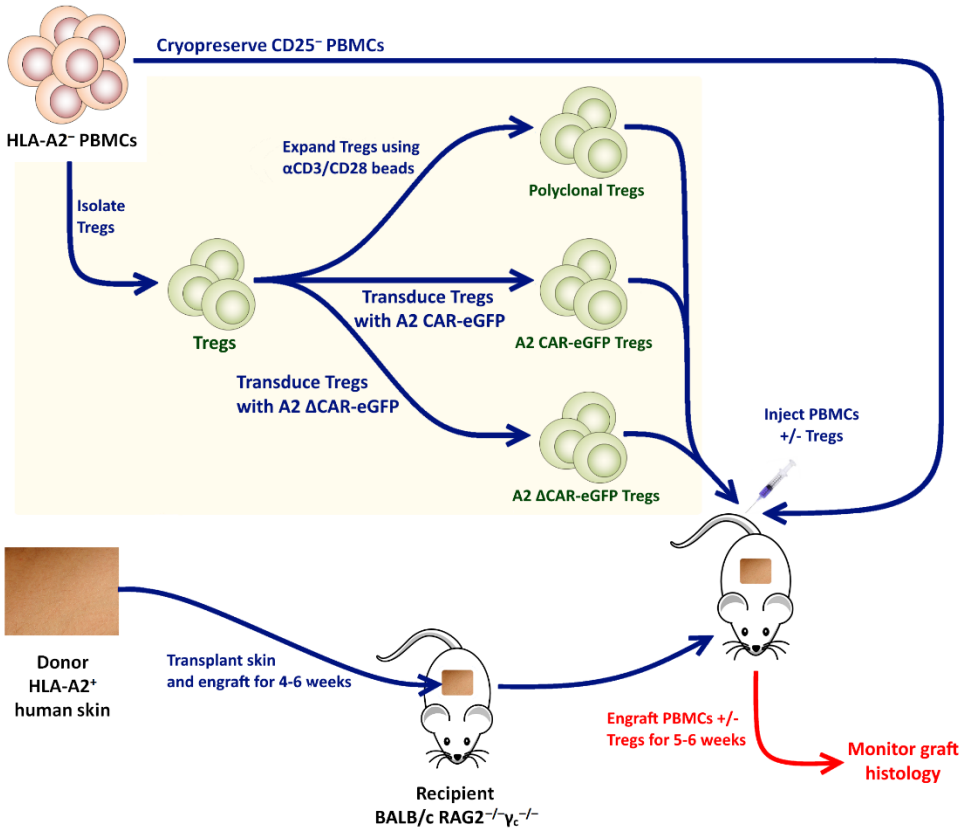

Figure 2-4 | **Summary of human skin xenograft transplant model.** *Split thickness HLA-A2⁺ human skin grafts measuring approximately 1.5 cm^2 were transplanted onto the back of recipient BALB/c RAG2$^{-/-}$γc$^{-/-}$ mice and allowed to engraft for 4-6 weeks. 100 µg anti-mouse Gr-1 was injected intraperitoneally every 3-4 days from this point onwards. During this engraftment period, allogeneic Tregs and PBMCs were isolated from a HLA-A2⁻ donor. PBMCs were depleted of CD25⁺ cells and cryopreserved. Tregs were transduced and expanded as previously described (Figure 2-1). BRG transplant recipients were then injected iv with 5x10^6 CD25⁻ PBMCs ± 1x10^6 expanded Tregs. Mice were sacrificed 5-6 weeks following PBMC transfer and skin grafts were harvested for histological analyses.*

Vetbond™ Tissue Adhesive (3M™, Maplewood, Minnesota, USA) and covered liberally with Fucidin® fusidic acid cream (LEO Pharma, Ballerup, Denmark). Two dressings were then applied: an OpSite® Spray Dressing (Smith & Nephew plc, London, UK) followed by a Jelonet paraffin gauze dressing (Smith & Nephew plc). The graft and dressings were secured by wrapping the mice with a Tegaderm™ Transparent Film Dressing (3M™). Mice were administered 100 µg anti-mouse Gr-1 (BioXCell, West Lebanon, New Hampshire, USA) intraperitoneally (ip) every 3-4 days from this point onwards.

Allografts were allowed to engraft for 5-6 weeks, after which the mice were injected iv with $5x10^6$ $CD25^-$ PBMCs ± expanded $1x10^6$ Tregs (sections 2.2.2 and 2.2.3). Mice were monitored every 2-3 days for changes in skin allograft morphology and weighed weekly to detect signs of xeno-GvHD. Human immune cell engraftment was assessed in the peripheral blood by flow cytometry staining for human and mouse $CD45^+$ cells (section 2.5.1). Mice were culled five weeks following PBMCs/Tregs transfer at which point the spleen and skin allograft was harvested for measuring human $CD45^+$ cell engraftment and histological analysis, respectively. A summary of the experimental protocol is provided in Figure 2-4.

2.7.2.1 Human skin allograft histological analysis

Human skin allografts were analysed with the help of Dr Christina Philippeos (Centre for Stem Cells & Regenerative Medicine, King's College London, UK).

Skin allografts were explanted and frozen in optimum cutting temperature (OCT; Thermo Fisher Scientific) over dry ice. Skin sections were cut at a thickness of 8 and 16 µm using a cryostat, air-dried and fixed by incubating in 4% paraformaldehyde for 15 minutes at room temperature.

Specificity	Host species	Clone	Company
CD3	Rabbit	Polyclonal	DAKO
FOXP3	Mouse	259D/C7	BD Pharmingen
CD45	Mouse	HI30	eBioscience
Ki67	Rabbit	Polyclonal	Abcam
Involucrin	Mouse	CY5	Sigma-Aldrich
CD31	Rabbit	Polyclonal	Abcam

Table 2-6 | **Primary antibodies used for staining human skin allograft sections.**

Specificity	Host species	Fluorochrome	Company
Mouse	Donkey	Alexa Fluor®555	Thermo Fisher Scientific
Rabbit	Donkey	Alexa Fluor®647	Thermo Fisher Scientific

Table 2-7 | **Secondary antibodies used for staining human skin allograft sections.**

Sections were washed three times (5 minutes each) with PBS and incubated with a blocking solution (PBS containing 10% donkey serum, 0.1% fish skin gelatin, 0.1% Triton X-100 and 0.5% Tween-20 (all from Sigma-Aldrich)) for 1 hour at room temperature. The sections were then stained with the primary antibodies listed in Table 2-6 and incubated at overnight at 4°C. Sections were washed twice for 5 minutes and incubated with secondary antibodies detailed in Table 2-7 with 2 µg/mL 4-6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) for 2 hours at room temperature. Sections were then washed 3 times for 5 minutes and mounted with Fluorescence Mounting Medium (DAKO, Agilent Technologies, Santa Clara, California, USA). Slides were stored at 4°C until analysis. Images were acquired at x20 magnification using a C2+ point scanning confocal microscope (Nikon). Maximum intensity projection (MaxIP) images consisting of 10 z-stacks (1.1 µm apart) were acquired and analysed/quantified with Nikon Instrument Software (NIS) Elements and Fiji is just ImageJ (FIJI) imaging software (Leung, Maurer et al. 2013).

2.7.3 *IN VIVO* MOUSE TREG PROLIFERATION ASSAY

Resting Tregs were harvested 7 days post-stimulation and labelled with CTV, as detailed in section 2.6.1.1. Recipient mice were injected iv with $2x10^6$ cells/mouse. Mice were culled 72 hours post-injection, spleens were explanted and splenocytes were isolated (section 2.4.6). Dead cells were labelled as detailed in section 2.5.1 and proliferation was measured by flow cytometry, as detailed in section 2.6.1.1.

2.8 STATISTICAL ANALYSIS

Data shown is mean ± standard error (SEM) or mean ± standard deviation (SD). Details pertaining to the statistical test used in each experiment are provided in the accompanying figure legends. In brief, the tests used were either a two-tailed paired Student's *t*-test, a two-way analysis of variance (ANOVA) or a one-way ANOVA with a Tukey multiple comparison post-hoc test. Significance is denoted as follows: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

CHAPTER III

DESIGN, GENERATION AND VALIDATION OF A HUMAN CHIMERIC ANTIGEN RECEPTOR SPECIFIC FOR HLA-A2

3.1 INTRODUCTION

3.1.1 DESIGNING A CAR

Over the past 25 years, CARs have evolved in a divergent manner to include elements from an ever-increasing number of proteins (section 1.3.1). A prime example of this is how the signalling domain of CARs has developed through multiple generations. This has given rise to a range of options which are available when designing a CAR with no universally accepted "optimal CAR design". As such, in addition to deciding what antigen to target, one must now consider factors such as how this antigen is to be targeted, which transmembrane domain to include, what generation of CAR to use or which signalling elements are to be incorporated with no consensus currently existing to address these aspects.

Despite this range of factors, two fundamental and overriding decisions must be made before anything else: i) what antigen is to be targeted, and; ii) what generation CAR is to be generated. HLA-A2 was selected as a suitable HLA class I molecule to target due to the high prevalence of this allele (>40%) in the UK population (Burt, Cryer et al. 2013). This simplified the identification of a HLA-A2 targeting moiety (Watkins, Brown et al. 2000) and well as the application of this CAR in various downstream experiments which required HLA-A2^{+} and HLA-A2^{-} donors. To address the second decision, a second generation CAR was selected based on the superior function of second generation CARs relative to first generation CARs (Finney, Lawson et al. 1998, Maher, Brentjens et al. 2002, Savoldo, Ramos et al. 2011). Furthermore, with second generation CARs proving adequate in multiple studies and clinical trials (Srivastava and Riddell 2015, Abate-Daga and Davila 2016), it was difficult to argue the benefits of using a third generation CAR, in this setting. In a cancer setting, additional signalling domains may be advantageous (Pule, Straathof et al. 2005, Hombach, Heiders et al. 2012, Srivastava and Riddell 2015, Abate-Daga and Davila 2016). However, given the results of Hombach *et al*. which demonstrated that the inclusion of two costimulatory domains (CD28 and OX40) abrogated CAR T cell production of the anti-inflammatory cytokine IL-10, for the purpose of generating CAR Tregs, a second generation CAR may in fact be preferable (Hombach, Heiders et al. 2012). Consequently, bearing in mind the importance of CD28 for Treg activation (Hombach, Kofler et al. 2007), a CAR was designed to incorporate a CD28-CD3ζ signalling domain.

Having decided which components to collate into a CAR, the next question to address was how this CAR was to be delivered in to human T cells and Tregs. The most prevalent approach which has been used in previous studies and CAR-based clinical trials to deliver genes of interest (GOI) into target cells is to use retroviruses.

3.1.2 RETROVIRUSES IN RESEARCH AND THE CLINIC

Retroviruses (*Retroviridae*) are a special family of virus which are able to integrate proviral DNA into the genome of an infected cell. This has made them fundamental tools in biological research where they are commonly used to deliver GOIs into target cells.

The retroviral virion is comprised of three key components: an envelope, various proteins and the RNA genome. The envelope is a lipid bilayer casing which is formed when the virus "buds" out of the host cell. This contains a protein capsid which houses the RNA genome and fundamental proteins such as the enzymes reverse transcriptase and integrase which are discussed below.

Virions infect target cells through the use of specific glycoproteins which are present on the outer surface of the envelope lipid bilayer. These glycoproteins are encoded by specific *env* genes which differ between viral species and dictate which target cells are infected. For example, the viral haemagglutinin (HA) present on the surface of most human influenza strains binds the *N*-acetylneuraminic acid-α2,6-galactose (NeuAcα2,6Gal) linkage on sialyloligosaccharides (Rogers and Paulson 1983, Rogers, Pritchett et al. 1983), sugar groups which is present at high levels on upper respiratory tract epithelial cells (Baum and Paulson 1990, Couceiro, Paulson et al. 1993, Gagneux, Cheriyan et al. 2003). Alternatively, the glycoprotein (gp)120 on the surface of the human immunodeficiency virus (HIV) preferentially binds the CD4 co-receptor of T cells (Yoon, Fridkis-Hareli et al. 2010). This viral specificity, termed "viral tropism", can be exploited experimentally to generate viral particles which preferentially infect certain cell types, a process is termed "pseudotyping".

Engagement of the virion glycoproteins with cognate cell surface receptors facilitates fusion of the lipid bilayer envelope with the plasma membrane of the target cell and release of the nucleocapsid (capsid containing RNA) into the cytosol (Nisole and Saib 2004, Grove and Marsh 2011). The precise mechanisms of the subsequent processes remain a matter of debate, particularly with regards to timing and location (Iordanskiy and Bukrinsky 2007, Arhel 2010, Campbell and Hope 2015), but the overall outcome is that the protein capsid uncoats to release its contents whilst the RNA is reverse transcribed. For the latter, single-stranded RNA molecules, which form the genome of the virion, are incorporated into reverse transcription complexes (RTC). RTCs utilise the enzyme reverse transcriptase (encoded by *pol*) to synthesise proviral DNA from the RNA template. This DNA combines with other proteins such as the enzyme integrase (also encoded by *pol*) to form pre-integration complexes (PIC) which facilitate entry of the proviral DNA into the nucleus (Arhel 2010, Eckwahl, Telesnitsky et al. 2016).

Nuclear access of the PIC typically relies on the breakdown of the nuclear membrane which occurs during cell division (Campbell and Hope 2015). As such, typical gamma-retroviruses do not efficiently integrate genes into the genomes of target cells which are not actively proliferating (Yamashita, Perez et al. 2007, Campbell and Hope 2015). However, lentiviruses, a specific genera of retrovirus, form PICs which are able to efficiently infect non-dividing cells as these PICs enter the nucleus through nuclear pores without disrupting the nuclear membrane (Piller, Caly et al. 2003, De Rijck, Vandekerckhove et al. 2007). Consequently, to efficiently deliver GOIs into target cells which do not rapidly proliferate, a lentiviral approach is typically preferred.

Once in the nucleus, integrase helps incorporate the proviral DNA into the genomic DNA. Consequently, progeny cells derived from the transduced cells contain the GOI which is of particular use and great importance when generating a T cell or Treg line that is being expanded *ex vivo* for clinical use. However, despite this advantage, cell therapy using genetically modified cells is regarded as a potentially hazardous process as retroviruses integrate their genes into genomic DNA is a partially random manner with active regions of the genome being preferred integration sites (Schroder, Shinn et al. 2002, Wu, Li et al. 2003, Kvaratskhelia, Sharma et al. 2014, LaFave, Varshney et al. 2014). Consequently, there is a potential to inadvertently activate proto-oncogenes or disrupt essential regulatory processes in target cells, resulting in the generation of cancerous cells.

In spite of these concerns, the clinical use of retroviral and lentiviral vectors has prevailed. In fact, trials using CAR modified T cells have now been performed for >10 years (Kershaw, Westwood et al. 2006) with no adverse effects due to insertional mutagenesis being observed during this time. However, the aforementioned concerns are not misplaced as serious adverse effects have been observed in alternative settings, most notably in X-linked SCID (SCID-X1) patients. These patients lack the γ_c gene thus in these trials, patients were administered autologous $CD34^+$ stem cells which were modified using retroviruses to express the γ_c. Gene insertion was confirmed to principally occur in or near genes that were highly expressed in the $CD34^+$ cells (Aiuti, Cassani et al. 2007, Deichmann, Hacein-Bey-Abina et al. 2007, Schwarzwaelder, Howe et al. 2007). In the first two trials, insertional mutagenesis led to the development of T cell leukaemias in 2/10 (Hacein-Bey-Abina, Von Kalle et al. 2003) and 4/9 (Hacein-Bey-Abina, von Kalle et al. 2003) patients treated, with similarly concerning results being obtained in subsequent trials (Deichmann, Hacein-Bey-Abina et al. 2007, Howe, Mansour et al. 2008).

In the laboratory, various steps have been adopted to improve the safety of *in vitro* virus production, particularly with regards to lentiviral production (Cepko and Pear 2001). A key step was to split the fundamental *gag*, *pol* and *env* genes into separate vectors which has given rise to

a number of different generations of lentiviral vectors. In current research, vectors of at least the second generation are used whereby packaging cells must be transfected with three separate plasmids: i) an expression vector containing the GOI; ii) a packaging plasmid containing the *gag-pol* polyprotein gene, and; iii) an envelope plasmid containing an *env* gene.

3.2 AIMS AND OBJECTIVES

The primary aim of this chapter was to design and generate a second generation CAR which was specific for HLA-A2 and comprised a human CD28-CD3ζ signalling domain. The functionality and specificity of this CAR was validated through the use of a previously established Tconv assay (Parente-Pereira, Wilkie et al. 2014) and different transduction approaches were explored to identify an optimal method for delivering the A2 CAR gene into target cells.

3.3 RESULTS

3.3.1 DESIGN AND GENERATION OF A HUMAN CAR SPECIFIC FOR HLA-A2

3.3.1.1 Design of a HLA-A2-targeting moiety

In 2000, Watkins *et al.* identified a 57 year old female patient who developed post-transfusion purpura (PTP) 5 days following a routine blood transfusion (Watkins, Brown et al. 2000). PTP manifests as a rash on the skin and is caused by the generation of allospecific antibodies which opsonise transferred platelets, leading to thrombocytopenia (Greenway 1991). Subsequent investigations performed on the serum of this patient identified the presence of antibodies specific for HLA-A2.

The authors proceeded to use "phage display" to obtain the sequences of novel scFv clones specific for HLA-A2. Mononuclear cells were isolated from the patient, total RNA was extracted and IgG variable heavy (V_H) chain sequences were amplified by PCR. The variable light (V_L) chain sequences used were obtained from an irrelevant, previously established scFv phage display library (Marks, Hoogenboom et al. 1991). These V_L chain sequences had previously been cloned into pHEN vectors (Sheets, Amersdorfer et al. 1998). As such, scFv open reading frames (ORF)

were generated by cloning the patient-derived V_H chain sequences into the donated pHEN-V_L-rep vectors, resulting in an scFv library consisting of $4.7x10^7$ clones.

Bacteriophage particles displaying the various scFv clones were co-cultured with HLA-$A2^+$ platelets for 2 hours, after which the platelets were thoroughly washed. The platelets were then incubated with TG1 *E. coli*. This allowed the phage still bound to the platelets to infect the *E. coli*. Transduced *E. coli* clones were then stimulated to secrete soluble scFv and the ability of these scFvs to bind HLA-A2 was assessed by staining HLA-$A2^+$ platelets or lymphocytes and using flow cytometry (termed a platelet/lymphocyte immunofluorescence test (PIFT or LIFT) (von dem Borne, Verheugt et al. 1978)). Three similar HLA-A2-specific scFv sequences were identified in this process. For this thesis, the published 3PB2 V_H chain and DPK1 V_L chain sequences were used to construct a HLA-A2-specific scFv. The specificity of this scFv was validated using immunoprecipitation by Dr Mohammad Ibrahim (Department of Allergy and Clinical Immunology, King's College Hospital, UK; unpublished data).

A HLA-A2-specific CAR antigen-targeting domain was designed using the aforementioned HLA-A2-specific scFv sequence, as shown in Figure 3-1. In this sequence, the V_H was linked to the downstream V_L through a $(GGGGS)_3$ serine/glycine linker. A human CD8α signal peptide sequence was added upstream of the scFv sequence to ensure that following expression, the CAR was translocated to the cell membrane. Additionally, NcoI and NotI restriction sites were added to the 5' and 3' ends of this antigen-binding domain sequence, respectively, to facilitate cloning. This sequence was then gene synthesised by GenScript (Piscataway Township, New Jersey, USA).

3.3.1.2 Construction of a human HLA-A2-specific CAR

Following validation of the nucleotide sequence, the scFv-coding DNA was cloned into the SFG retroviral expression vector which was kindly donated by Dr Michel Sadelain (Memorial Sloan Kettering Cancer Center, New York, USA). This vector had previously been modified by Dr John Maher (Division of Cancer studies, King's College London, UK) to contain the ORF of a second generation CAR (Maher, Brentjens et al. 2002). The extracellular antigen targeting moiety of this CAR contained an NK1 sequence (Thayaparan, Maher, unpublished data), a naturally-occurring splice variant of the ligand hepatocyte growth factor (Jakubczak, LaRochelle et al. 1998). This was followed by a 9E10 cMyc epitope, a human CD28 hinge/transmembrane domain and a human CD28-CD3ζ signalling domain.

The NK1-targeting moiety of the NK1-28ζ CAR was replaced with the HLA-A2-targeting moiety described in Figure 3-1A, using the unique 5' NcoI and 3' NotI restriction sites shown in Figure 3-2A. This cloning process was performed by GenScript and resulted in the generation of an SFG retroviral vector containing the ORF of a HLA-A2-specific CAR comprising a CD28-CD3ζ signalling domain, hereon in referred to as "SFG_A2-28ζ". Upon receipt of this SFG_A2-28ζ vector, the results of the gene synthesis and cloning process were validated in house by restriction digestion using NcoI and XhoI restriction enzymes. Two fragments of 6.4 and 1.5 kilobases (kbp) were expected in order to confirm that the scFv was correctly orientated in the SFG retroviral vector. Bands corresponding to DNA fragments of these sizes were identified following the digestion (Figure 3-3).

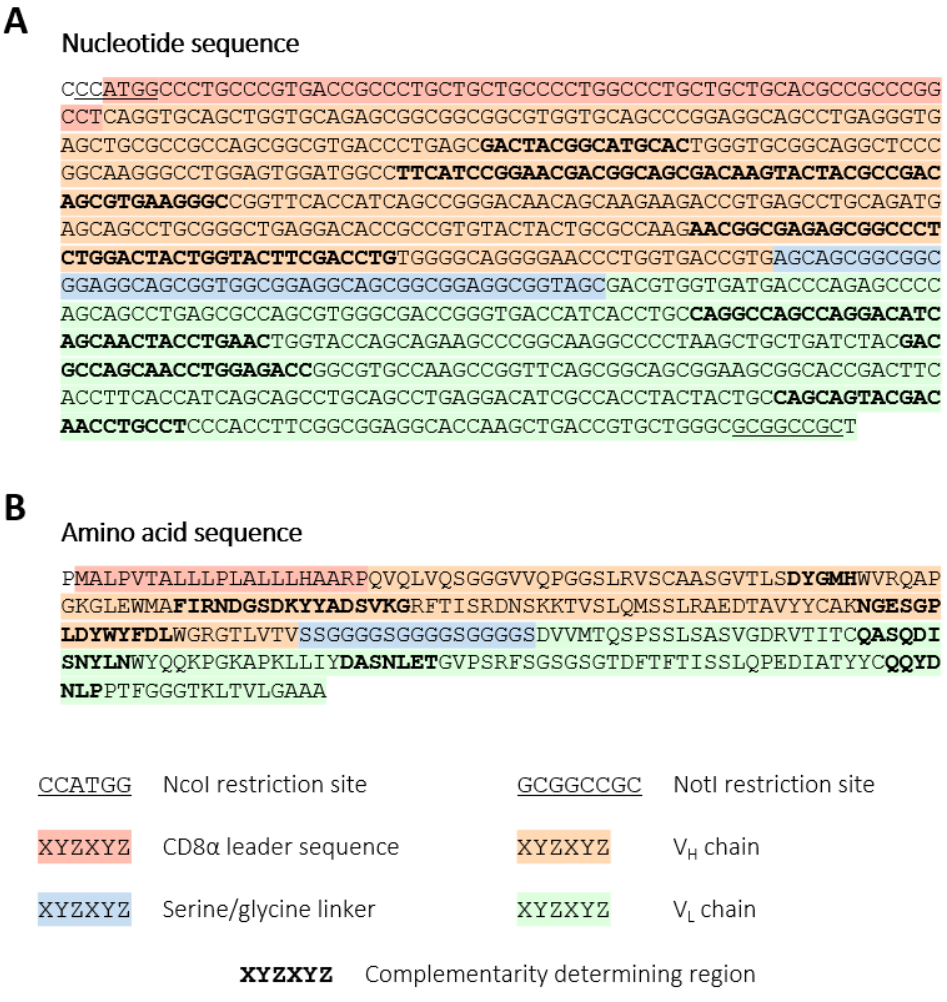

Figure 3-1 | **Diagram detailing the components of the HLA-A2-targeting moiety used for constructing a HLA-A2-specific CAR.** *A: Nucleotide sequence of the HLA-A2-targeting moiety. Flanking 5' NcoI and 3' NotI restriction sites are underlined. B: Amino acid sequence of the HLA-A2-targeting moiety. The sequence of a human CD8α leader (red), scFv V_H chain (orange), serine/glycine linker (blue) and scFv V_L chain (green) are shown in addition to predicted complementarity determining regions (CDR) in bold.*

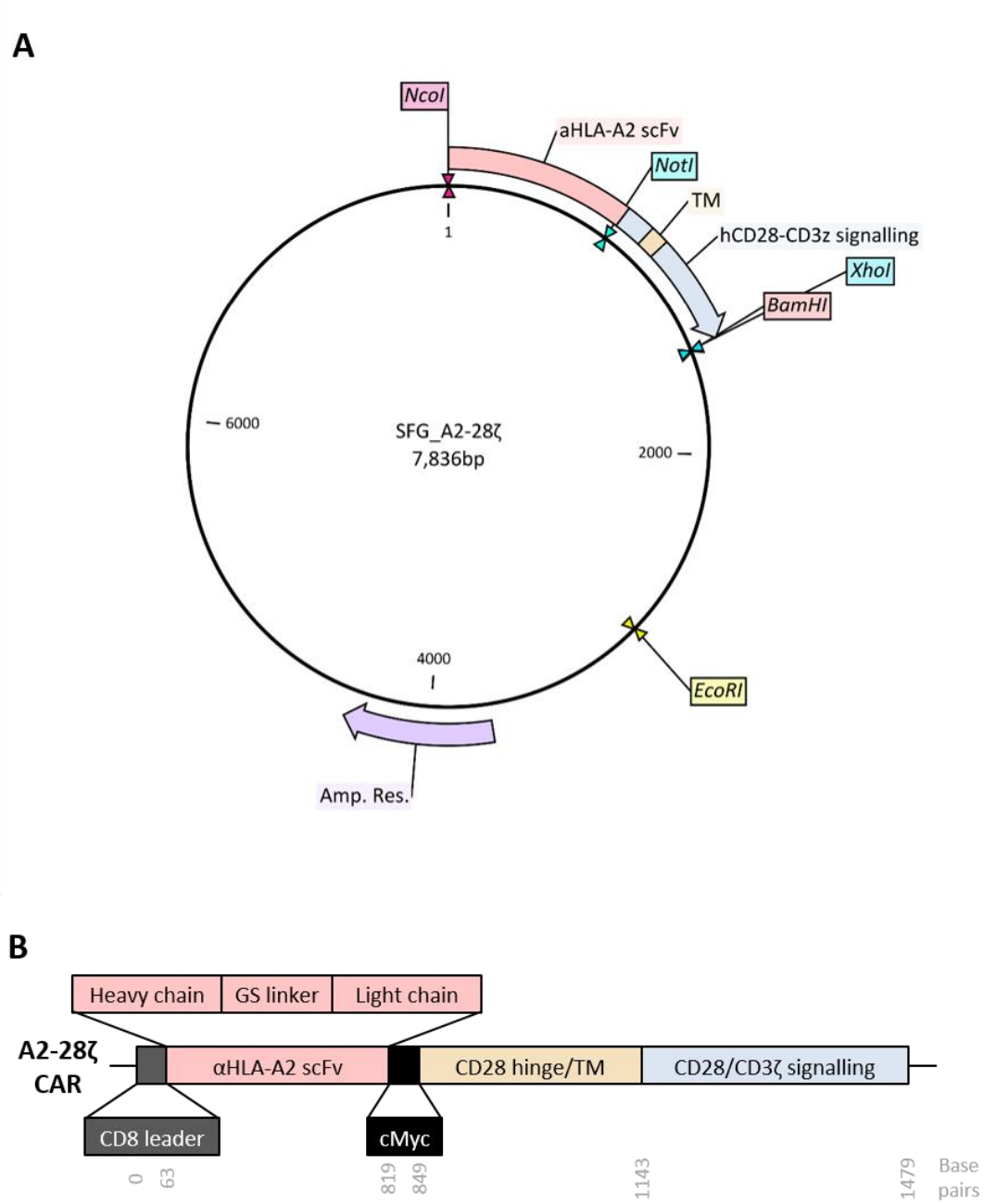

Figure 3-2 | **Restriction map of the SFG_A2-28ζ plasmid and a schematic diagram of the A2-28ζ CAR.** *A: Restriction map of the SFG retroviral expression vector containing the A2-28ζ CAR gene. The HLA-A2-targeting sequence (red) was cloned between NcoI and NotI to yield the sequence of a HLA-A2-specific second generation CAR comprising a human CD28 transmembrane domain (orange) and a CD28-CD3ζ signalling domain (blue). The complete nucleotide sequence of this vector is provided in the Supplementary data, Figure S2. B: Schematic diagram of the A2-28ζ CAR. A preceding human CD8α sequence (grey) was added upstream of the HLA-A2-targeting moiety (red) to facilitate trafficking of the CAR protein to the cell membrane. The MYPPPY recognition motif in the CD28 extracellular region was replaced with a 9E10 cMyc epitope (black), downstream of which was a human CD28 hinge/transmembrane domain (orange) and CD28-CD3ζ signalling domain (blue). Amp. Res. = ampicillin resistance, TM = transmembrane.*

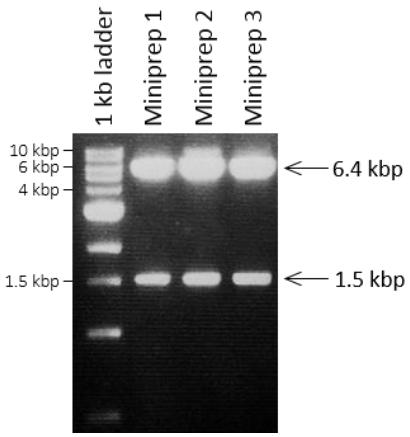

Figure 3-3 | **Restriction digest of SFG_A2-28ζ plasmid minipreps with NcoI and XhoI.** *DNA isolated from 3 minipreps of the SFG_A2-28ζ were restriction digested with NcoI and XhoI. Resultant DNA fragments were separated on a 1% agarose gel with a 1 kb DNA ladder.*

3.3.2 Functional validation of the A2 CAR

The functionality and specificity of the HLA-A2-specific CAR (A2 CAR) was assessed using a previously established Tconv functional assay (Parente-Pereira, Wilkie et al. 2014). In this experimental procedure, human PBMCs were activated with PHA-L and retrovirally transduced to express the A2 CAR. These cells were then co-cultured with tumour cell monolayers which were HLA-A2^{+} or HLA-A2^{-}. It was anticipated that if the A2 CAR was functional, the CAR T cells would be activated in the presence of HLA-A2^{+} tumour monolayers. This would result in the elicitation of cytotoxic activity and production of pro-inflammatory cytokines, a response which would not be expected in the absence of HLA-A2.

3.3.2.1 Retroviral transduction delivered the A2 CAR gene into unfractioned human T cells

As discussed in the chapter introduction, retroviral transduction is a well-established and safe approach for delivering genes of interest into target cells (Scholler, Brady et al. 2012). In order to deliver the A2 CAR gene into PBMCs, GaLV-pseudotyped retroviral particles containing the SFG_A2-28ζ construct were generated using a two-step process (Parente-Pereira, Wilkie et al. 2014). This two-step process was employed such that a stable cell line could be generated which consistently produced desired retroviral particles, eliminating the requirement to transfect fresh cells every time viral particles were desired. To generate this line, genomic integration of the A2 CAR gene was required. As such, it was necessary to transfect one retroviral packaging cell line to

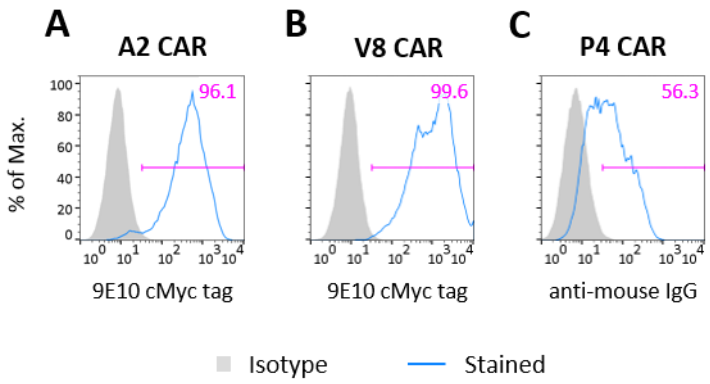

Figure 3-4 | **PG13 retroviral packaging cells are efficiently transduced with VSV-G-pseudotyped retroviral particles to express the A2, V8 and P4 CARs.** *PG13 cells were transduced with VSV-G-pseudotyped retroviral particles to express the A2 (A), V8 (B) or P4 (C) CAR. The proportion of PG13 cells which was successfully transduced was measured by flow cytometry. Expression of the A2 and V8 CARs was detected by staining for the 9E10 cMyc tag epitope whilst expression of the P4 CAR was detected using an anti-mouse IgG antibody which reacted with murine elements within the scFv (blue line). Cells stained with an isotype control are shown in solid grey. The percentage of cells in which CAR expression was detected, determined by the gate provided, is shown in the top right corner of each plot.*

transiently release VSV-G-pseudotyped viral particles which could then be used to transduce a second stable retroviral packaging cell line which secreted GaLV-pseudotyped viral particles.

Initially, the SFG_A2-28ζ construct was transfected into H29D human retroviral packaging cells (Ory, Neugeboren et al. 1996) using calcium phosphate (Graham and van der Eb 1973). These cells were cultured in the absence of tetracycline to allow VSV-G-pseudotyped retroviral particles to be produced (section 2.2.4). Viral supernatant was harvested from these H29D cells 5 days post-transfection and used to transduce PG13 cells. PG13 cells were incubated with H29D-derived viral supernatant for 72 hours and maintained in culture for at least one week prior to analysis.

In subsequent experiments, two control CARs previously constructed by Dr John Maher were used to confirm that recognition of HLA-A2$^+$ target cells was indeed dependent on the CAR possessing a HLA-A2-targeting moiety. Both of these CARs contained a CD28-CD3ζ signalling domain which matched that present in the A2 CAR. V8 was a control CAR of irrelevant antigen specificity which was generated by point-mutating the peptide-based antigen-targeting moiety of an integrin $\alpha_v\beta_6$-specific CAR (Whilding, Parente-Pereira et al. 2016) whilst P4 was a prostate-specific membrane antigen (PSMA)-specific CAR (Maher, Brentjens et al. 2002).

Expression of the A2, V8 and P4 CARs in transduced PG13 cells was measured by flow cytometry (Figure 3-4). A2 and V8 CARs were detected using an antibody specific for the 9E10 cMyc tag epitope present in the extracellular domain of these CARs (Figure 3-2B). However, as the P4 CAR did not contain a cMyc tag epitope, expression of this CAR was measured using an anti-mouse IgG antibody which coincidently recognised the murine scFv domain of the CAR. As shown in Figure 3-4, the A2 and irrelevant V8 CARs were expressed in PG13 cells with a high efficiencies of 96.1% and 99.6%, respectively. However, the proportion of PG13 cells expressing the irrelevant P4 CAR was lower (56.3%).

In order to assess whether GaLV-pseudotyped retroviral particles could deliver a GOI into target T cells, unfractioned human T cells from peripheral blood were activated and transduced with supernatant derived from the stably transduced PG13 cells. PBMCs were isolated from a HLA-A2^{-} donor and cultured with PHA-L (5 µg/mL) and IL-2 (100 U/mL) to selectively activate the T cells. The T cells were transduced to express the A2, V8 or P4 CARs after 72 hours by mixing the cells in viral supernatant derived from the PG13 cells, spinoculating the cells at 600 g for 1 hour in RetroNectin®-coated plates and incubating the cells at 37°C with 5% CO_2. Transduced cells were expanded for 7-10 days where fresh media and IL-2 was supplied every 2 days. The transduction efficiency was then assessed by flow cytometry and functional assays were performed. As shown

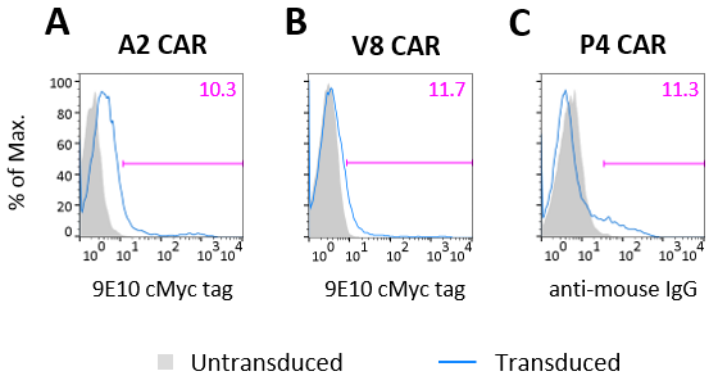

Figure 3-5 | **Unfractioned human T cells are successfully transduced with GaLV-pseudotyped retroviral particles to express the A2, V8 and P4 CARs.** *Human PBMCs were isolated from a HLA-A2^{-} donor, activated with PHA-L and retrovirally transduced with PG13-derived GaLV-pseudotyped retroviral particles to express the A2 (A), V8 (B) or P4 (C) CARs 72 hours post-activation. The transduction efficiency was measured 7-10 days post-transduction by flow cytometry, using antibodies specific for the 9E10 cMyc tag epitope (A2 and V8 CAR) or an anti-mouse IgG antibody (P4 CAR). Stained cells are shown with a blue line and isotype control staining is shown in solid grey. The percentage of CAR-expressing T cells, as determined by the gate shown, is provided in the top right corner of each plot. Data is representative of 4 individual experiments.*

in Figure 3-5, total T cells were successfully transduced to express the CARs with efficiencies of approximately 10% (A2 = 9.9 ± 7.6%, V8 = 11.6 ± 15.2% and P4 = 10.8 ± 10.8%; n=4). Overall, these results demonstrated that GaLV-pseudotyped retroviral particles delivered CARs into target T cells with a sufficient efficiency for downstream experiments to be performed.

3.3.2.2 Human T cells expressing the A2 CAR exhibited cytotoxicity in a HLA-A2-specific manner

A literature search was performed to identify adherent target cells which differentially expressed HLA-A2. Two breast cancer epithelial cell lines were identified (Carlsson, Forsberg et al. 2007) and kindly donated by Dr John Maher. In this study, MCF-7 cells were shown to express HLA-A2^{+} whilst T-47D cells were reported to be HLA-A2^{-}. These findings were confirmed by flow cytometry using 2 separate antibody clones (BB7.2 and REA142), as shown in Figure 3-6A-B. Furthermore, to confirm that the lack of HLA-A2 detection in the T-47D cells was due to these cells being negative for the expression of HLA-A2, and not because these cells downregulated HLA class I molecules, both cell lines were analysed for expression of total HLA class I (Tu149 clone) and HLA-DR (L243 clone). Interestingly, T-47D cells were found to express significantly more total HLA class I (mean fluorescence intensity (MFI) = 20,915), compared to the MCF-7 cells (MFI = 1,045; Figure 3-6C), whilst both cell lines expressed low levels of HLA-DR (Figure 3-6D). Overall, these results confirmed that MCF-7 cells expressed high levels of HLA-A2 whilst T-47D cells did not express this molecule, despite expressing high levels of total HLA class I.

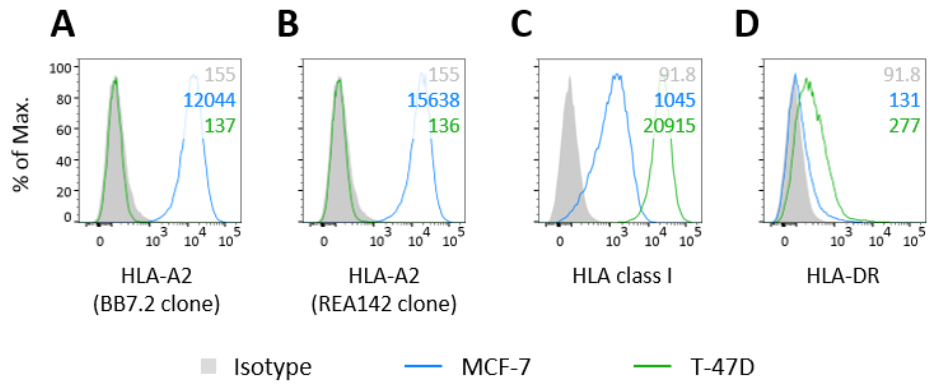

Figure 3-6 | **MCF-7 and T-47D differentially expressed HLA-A2.** *MCF-7 (blue line) and T-47D (green line) breast cancer epithelial cells were stained with fluorophore-conjugated antibodies to measure their expression of HLA-A2 (A/B) HLA class I (C) and HLA-DR (D) by flow cytometry. Two separate antibodies clones (BB7.2 and REA142) were used to confirm HLA-A2 recognition. Isotype control staining is shown in solid grey and MFI values are provided.*

To assess the functionality and specificity of the A2 CAR, unsorted transduced T cells were co-cultured overnight with confluent monolayers of MCF-7 and T-47D cells. Following co-culture, supernatants were collected to analyse cytokine production. Cell monolayers were washed gently using PBS to remove contaminating T cells and an MTT assay was performed to measure the viability of the cell monolayers.

Cytotoxicity was observed microscopically when T cells expressing the A2 CAR were co-cultured with HLA-A2^{+} MCF-7 cells (Figure 3-7A). In contrast, destruction of the monolayers was not observed when the A2 CAR T cells were co-cultured with HLA-A2^{-} T-47D cells, nor when T cells

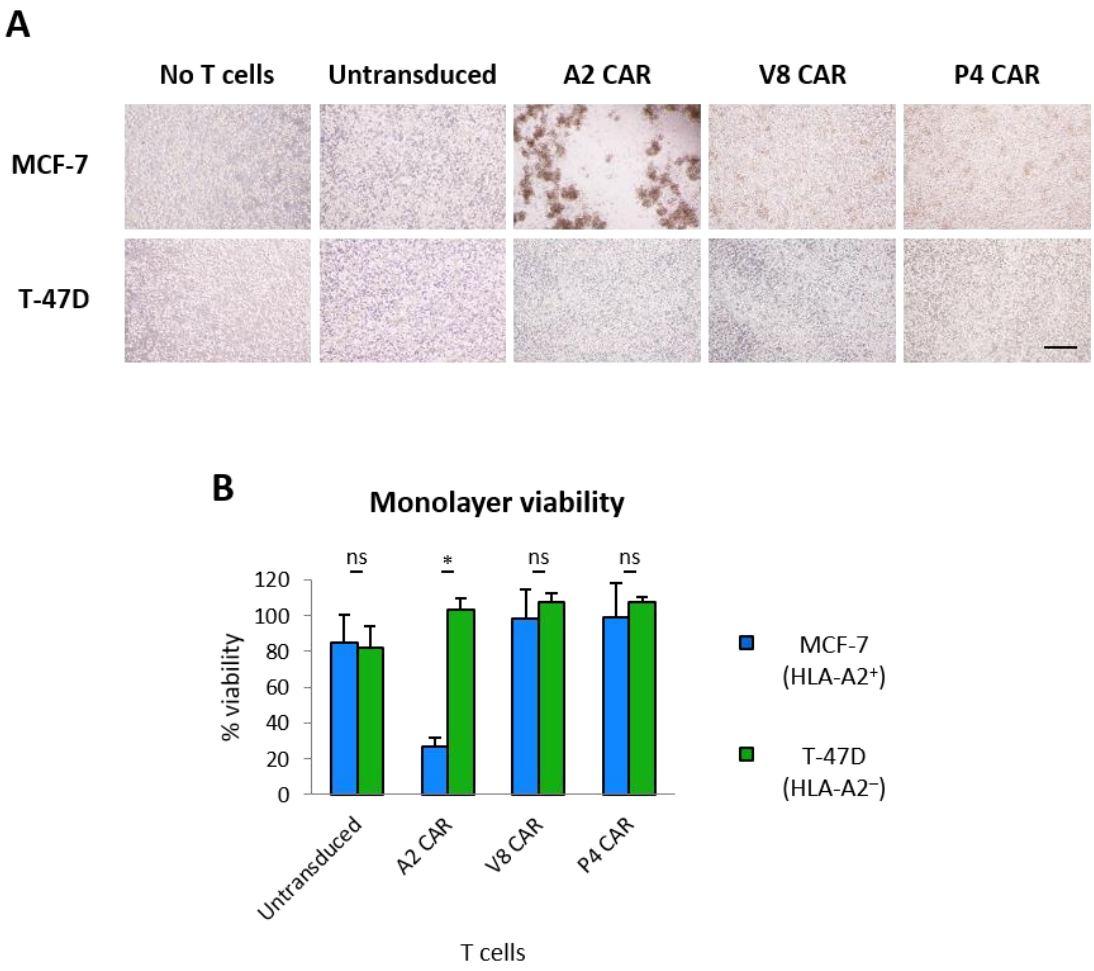

Figure 3-7 | **T cells transduced to express the A2 CAR exhibited cytotoxicity in a HLA-A2-dependent manner.** *A: CAR-modified T cells were co-cultured overnight with confluent monolayers of MCF-7 and T-47D cells. Images were taken after the co-culture at x10 magnification. Scale bar represents 250 µm. B: The viability of the cell monolayers following co-culture was quantified by MTT assay. % viability of MCF-7 (blue bars) and T-47D (green bars) cells is shown relative to monolayer cells cultured alone. Data represents mean + SEM pooled from 3 individual experiments. Significance was determined using a two-tailed paired Student's t-test where *=p<0.05. ns = not significant.*

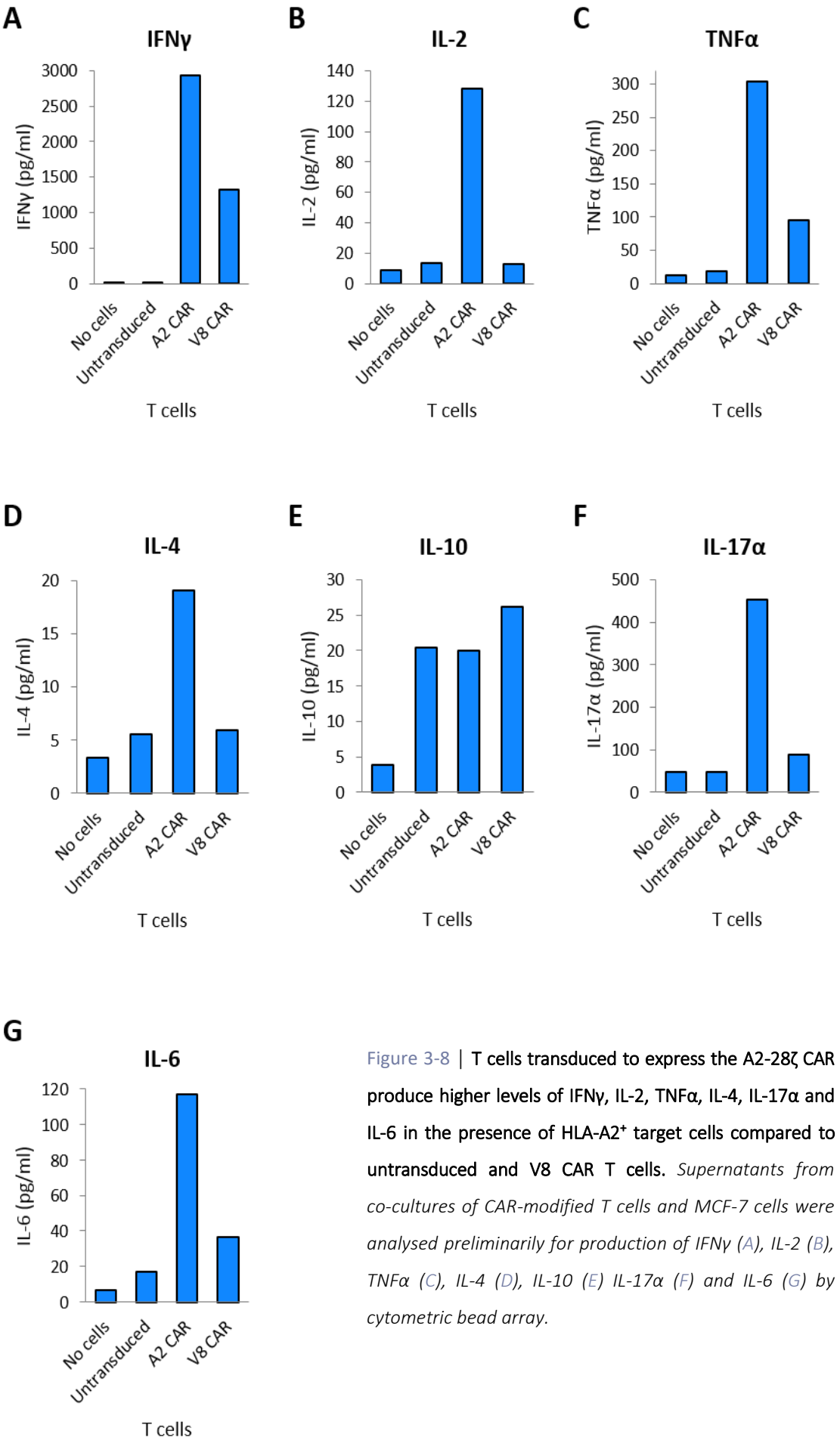

Figure 3-8 | **T cells transduced to express the A2-28ζ CAR produce higher levels of IFNγ, IL-2, TNFα, IL-4, IL-17α and IL-6 in the presence of HLA-A2⁺ target cells compared to untransduced and V8 CAR T cells.** *Supernatants from co-cultures of CAR-modified T cells and MCF-7 cells were analysed preliminarily for production of IFNγ (A), IL-2 (B), TNFα (C), IL-4 (D), IL-10 (E) IL-17α (F) and IL-6 (G) by cytometric bead array.*

expressing an irrelevant CAR were co-cultured with either of the cell lines. The viability of the MCF-7 cells was significantly lower (p=0.031) than the viability of the T-47D cells following co-culture with the A2 CAR T cells (MCF-7 = 27.0 ± 4.9%, T-47D = 103 ± 7.0%, n=3; Figure 3-7B). However, these differences in HLA-A2$^+$ and HLA-A2$^-$ monolayer viability were not observed following co-culture with the untransduced (p=0.40), V8 CAR (p=0.53) or P4 CAR (p=0.74) T cells. The efficacy of the A2 CAR T cells was particularly impressive given the fact that approximately 90% of these cells did not express the CAR construct (Figure 3-5A). Overall, these results demonstrated that T cells transduced to express the A2 CAR recognised HLA-A2 on target cells which led to elicitation of an effector function, in this case cytotoxicity.

To confirm these findings, co-culture supernatants were analysed for the presence of cytokines which are known to be released by T cells following activation. A preliminary cytometric bead array (CBA) analysis was performed to investigate the range of cytokines which were produced. This analysis included a measurement of IL-2, IFNγ, TNFα, IL-4, IL-10, IL-17α and IL-6 produced by T cells co-cultured with HLA-A2$^+$ MCF-7 cells only (Figure 3-8). From a single, preliminary experiment, A2 CAR T cells were shown to produce higher quantity of IL-2, IFNγ, TNFα, IL-4, IL-17α and IL-6 than untransduced or V8 CAR T cells. A2 CAR T cells production of IFNγ was 180-fold higher than untransduced T cells (Figure 3-8A). Similarly, the quantity of IL-2 (Figure

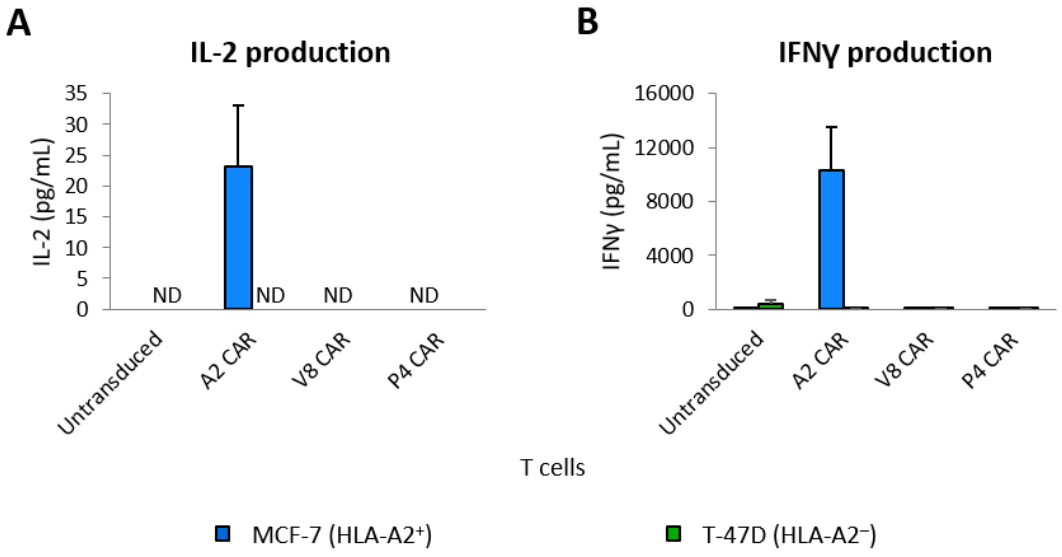

Figure 3-9 | **T cells transduced to express the A2 CAR produced high levels of IL-2 and IFNγ in the presence of HLA-A2$^+$ target cells.** *Supernatants from co-cultures of CAR-modified T cells and MCF-7 (blue bars) or T-47D (green bars) cells were analysed for production of IL-2 (A) and IFNγ (B) by cytokine-specific ELISAs. Data represents mean ± SEM pooled from 3 individual experiments. A two-tailed paired Student's t -test was performed but no significant differences were observed.*

3-8B), TNFα (Figure 3-8C) and IL-17α (Figure 3-8F) produced by the A2 CAR T cells was >9-fold more than untransduced T cells. Interestingly, V8 CAR T cells also produced noticeably higher levels of IFNγ and TNFα than untransduced T cells. However, this was likely an anomalous result as elevated IFNγ production from the V8 CAR T cells was not observed in subsequent experiments (measured by ELISA analysis; Figure 3-9).

IL-2 and IFNγ produced during the co-cultures was further analysed using cytokine-specific ELISAs. Using this approach, IL-2 production was only detectable when A2 CAR T cells were co-cultured with HLA-A2$^+$ MCF-7 cells (Figure 3-9A). Under the same conditions, these cells also produced high levels of IFNγ. A2 CAR T cells produced 212-fold more IFNγ when co-cultured with the HLA-A2$^+$ MCF-7 cells (10,341 pg/mL), compared to the HLA-A2$^-$ T-47D cells (48.8 pg/mL). This result did not reach statistical significance (p=0.11; n=3), likely due to a combination of variability in the donors and transduction efficiencies. In these assays, the untransduced, V8 CAR and P4 CAR T cells all produced low levels of IFNγ in the presence of the HLA-A2$^+$ MCF-7 cells (all <100 pg/mL). However, these findings clearly affirmed the functionality of the CAR, particularly given the fact that only approximately 10% of these cells expressed the CAR. Overall, these results demonstrated that T cells modified to express the A2 CAR were capable of functioning in a HLA-A2-specific manner, confirming the functionality and specificity of the novel CAR.

3.3.3 Sub-cloning of the HLA-A2-specific CAR ORF from a retroviral construct into a lentiviral construct

In order to aid downstream experiments, it was desirable to improve the efficiency with which T cells were transduced. As discussed in the introduction (section 3.1.2), lentiviruses are a subclass of retrovirus which are capable of transducing target cells in the absence of cell division (Cooray, Howe et al. 2012). As such, to investigate whether the T cell transduction efficiency could be improved by using a lentiviral approach, the A2 CAR gene was sub-cloned into a pLNT/SFFV_eGFP lentiviral vector (kindly provided by Dr Gilbert Fruhwirth; Division of Imaging Sciences and Biomedical Engineering, King's College London, London, UK).

The pLNT/SFFV second generation lentiviral vector was originally a kind gift provided by Prof Adrian Thrasher (Molecular and Cellular Immunology, University College London, UK). This vector was modified by Dr Gilbert Fruhwirth who inserted a cassette coding a short, flexible linker (GGAGGTGGCGCCACC) followed by the ORF of an enhanced green fluorescent protein (eGFP) reporter, resulting in the generation of the pLNT/SFFV_eGFP plasmid (Figure 3-10A).

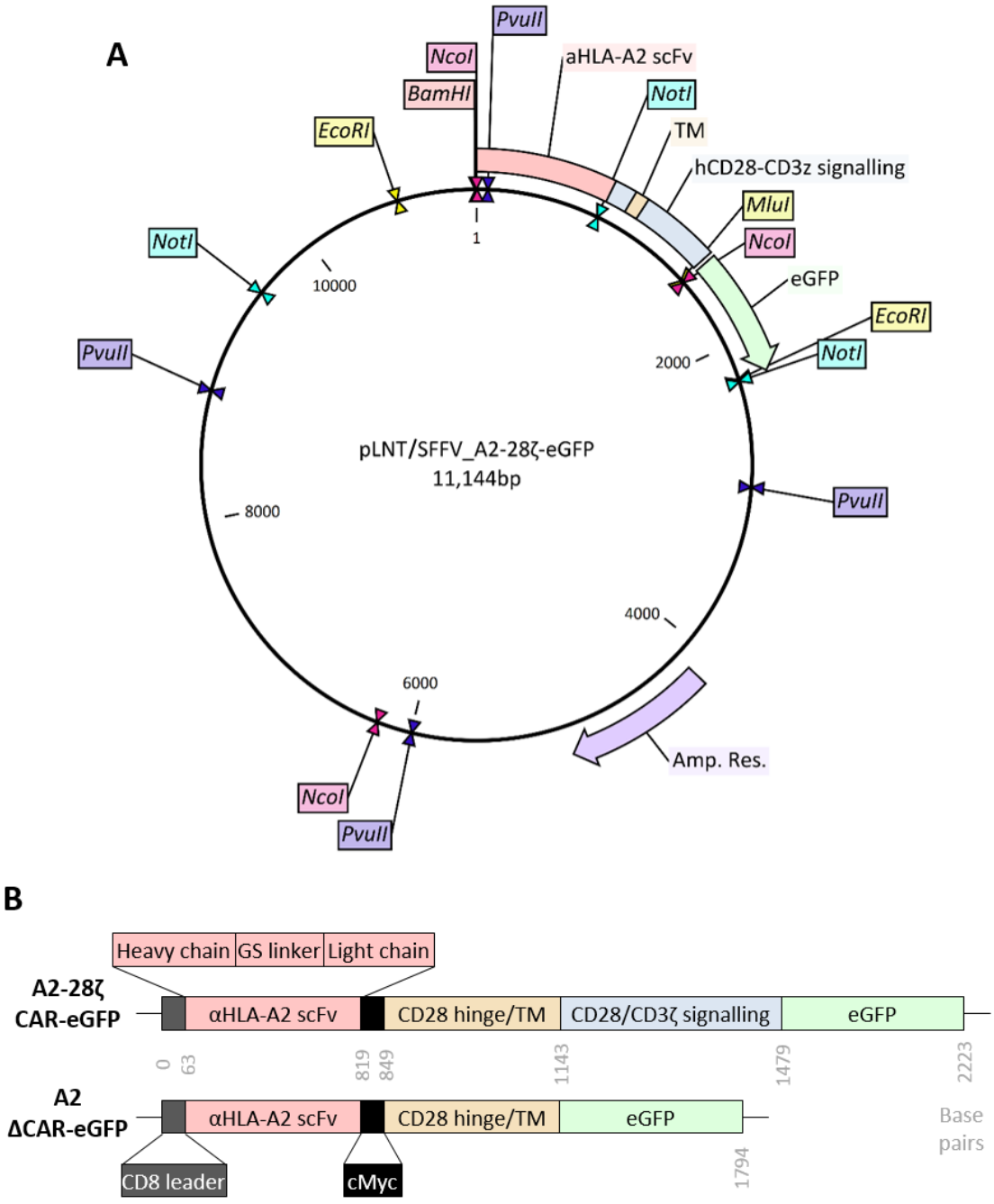

Figure 3-10 | **Restriction map of the pLNT/SFFV_A2 CAR-eGFP plasmid and a schematic diagram of the A2-28ζ CAR-eGFP and A2 ΔCAR-eGFP constructs.** *A: Restriction map of the pLNT/SFFV_eGFP lentiviral expression vector containing the full-length A2-28ζ CAR-eGFP ORF. The A2-28ζ CAR ORF (red/blue) was cloned between BamHI and MluI, immediately upstream of the eGFP ORF (green) to yield a CAR-eGFP fusion. The complete nucleotide sequence of this vector is provided in the Supplementary data, Figure S3. B: Schematic diagrams of the full-length A2-28ζ CAR-eGFP and truncated A2 ΔCAR-eGFP proteins. The complete nucleotide sequence of this vector is provided in the Supplementary data, Figure S1. Amp. Res. = ampicillin resistance.*

The aim of this cloning process was to insert the A2 CAR gene into the LNT/SFFV_eGFP vector immediately upstream of the eGFP ORF to generate a A2 CAR-eGFP fusion (Figure 3-10B). To assess whether a simple cloning strategy could be employed to achieve this, the flanking restriction sites of the A2 CAR gene in the SFG retroviral construct were compared to those present in the recipient pLNT/SFFV_eGFP construct but unfortunately, no matching sites were identified. Consequently, the cloning strategy shown in Figure 3-11 was employed.

PCR primers were designed to amplify the A2 CAR gene from the SFG retroviral construct whilst concurrently adding a BamHI and MluI restriction site to the 5' and 3' ends of the PCR products, respectively (Figure 3-12A and Table 3-1). Inserting the A2 CAR gene into the pLNT/SFFV_eGFP

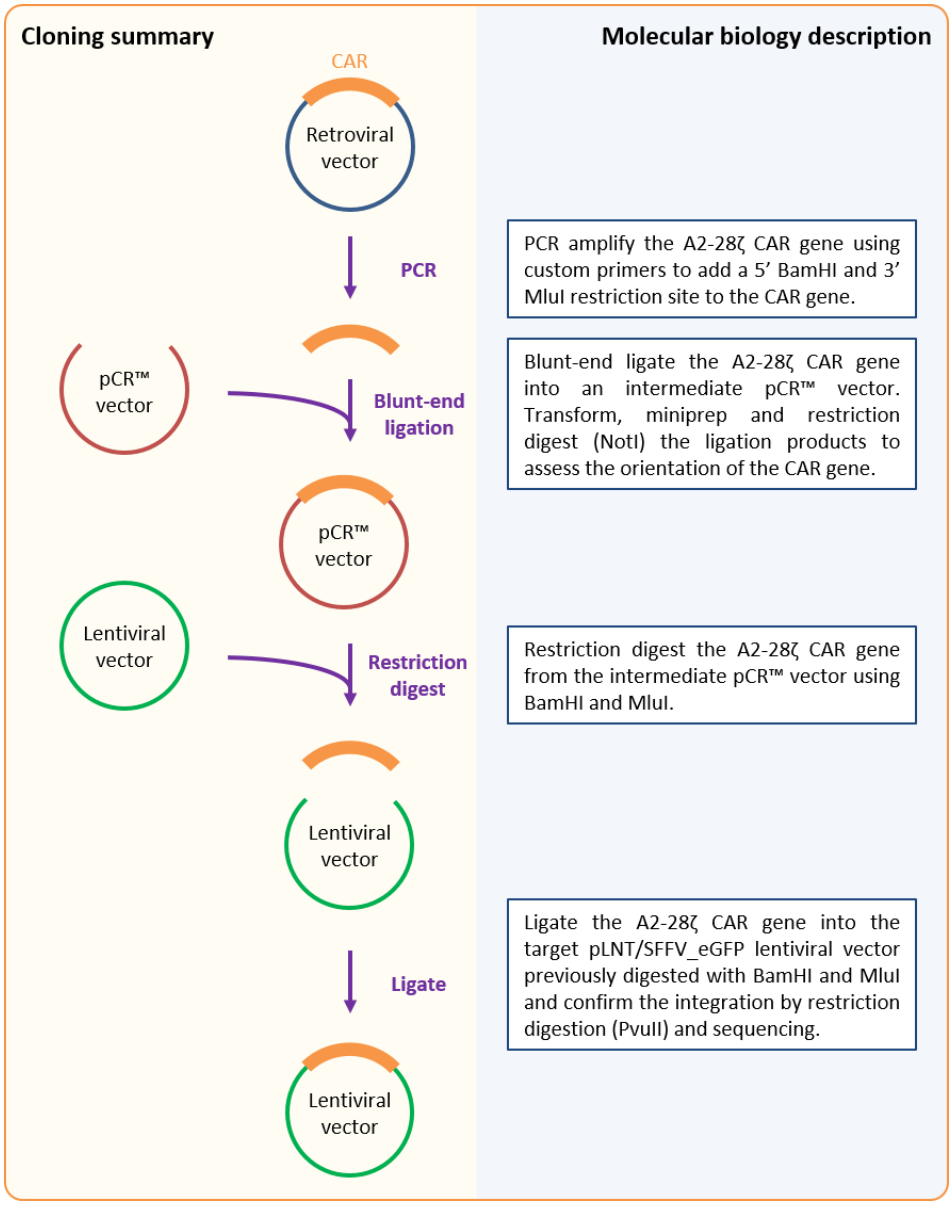

Figure 3-11 | **Summary of the cloning strategy employed to sub-clone the A2-28ζ CAR gene from the resident SFG retroviral construct into a recipient pLNT/SFFV_eGFP lentiviral construct.**

vector using the BamHI and MluI restriction sites (Figure 3-10A) would have resulted in the generation of an A2-28ζ-eGFP fusion ORF. However, as it was unclear whether the presence of a C-terminal eGFP fusion would influence the function of the CAR, a second reverse primer was designed whereby a TAA stop codon and an extra nucleotide was incorporated to prevent transcription of the downstream eGFP gene (Figure 3-12B and Table 3-1). Additionally, as this cloning process presented the opportunity to generate a truncated HLA-A2-specific CAR as an additional control, similar reverse primers were designed which recognised the A2-28ζ CAR

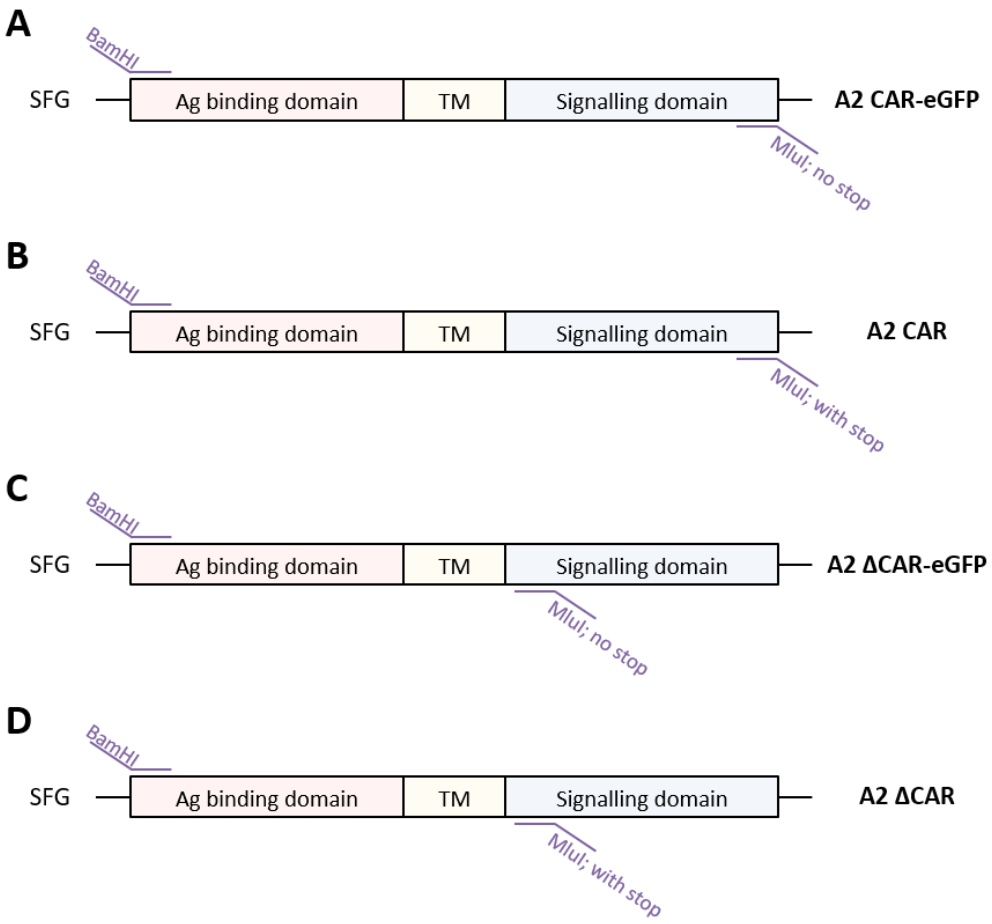

Figure 3-12 | **Schematic diagrams of custom PCR primers for amplifying the A2 CAR gene whilst adding a 5' BamHI and 3' MluI restriction site.** *Custom PCR primers were designed to amplify the A2-28ζ CAR gene whilst concurrently adding restriction sites to facilitate subsequent cloning. A universal primer recognising the 5' end of the start of the CAR was designed to add a BamHI restriction site and 4 reverse primers were designed to generate different CAR genes with a 3' MluI restriction site: a full-length CAR with a C-terminal eGFP fusion (A), a full-length CAR with no eGFP fusion (B), a truncated CAR with a C-terminal eGFP fusion (C) and a truncated CAR with no eGFP fusion (D).*

sequence in the CD28 signalling domain, upstream of the first ITAM, ultimately resulting in CARs which were capable of binding HLA-A2 but not signalling intracellularly (Figure 3-12C/D).

The designed PCR primers (Table 3-1) were synthesised by Sigma-Aldrich and used to amplify the A2 CAR gene. The forward primer was designed to recognise the first 19 nucleotides of the CD8α leader sequence whilst the reverse primers were designed to recognise either the last 19 nucleotides of the CD3ζ signalling domain (full-length CAR) or a 22 nucleotide region downstream of the transmembrane domain (truncated CAR; precise recognition loci are shown in the Supplementary data, Figure S1). These PCR primers were used to amplify the A2 CAR ORF from the resident SFG retroviral vector. Unidentified nucleic acid contaminants were removed from the PCR products using a membrane-based purification system (Figure 3-13A, red boxes) and the PCR products were blunt-end ligated into an intermediate pCR™ vector to facilitate an efficient downstream BamHI/MluI restriction digestion. Successfully ligated pCR™ vectors containing DNA inserts were expanded by transforming DH5α cells and growing these cells in the presence of kanamycin which inhibited the growth of *E. coli* that did not contain a pCR™ vector. DNA was isolated from single colonies by miniprep and pCR™ vectors containing a CAR insert in the correct orientation were identified by restriction digestion using NotI. Bands of 720 bp (complete CAR) and 300 bp (truncated CAR) were indicative of the CAR gene insert being orientated correctly (Figure 3-14, highlighted with green boxes) whilst bands of 865 bp (complete CAR) and 900 bp (truncated CAR) indicated the CAR gene insert was orientated incorrectly (Figure 3-14, highlighted with red boxes).

The pCR™ vectors containing correctly orientated CAR inserts and the pLNT/SFFV_eGFP lentiviral vector were restriction digested with BamHI and MluI to generate DNA fragments with flanking

Primer name	Sequence (5'-3')
Universal forward primer	GCGCGGATCCACCGCCATGGCCCTGCCCGTGACCG
Reverse primer for A2 CAR-eGFP	GCCGACGCGTGCGAGGGGGCAGGGCCTGC
Reverse primer for A2 CAR	GCCGACGCGTGTTAGCGAGGGGGCAGGGCCTGC
Reverse primer for A2 ΔCAR-eGFP	GCCGACGCGTGCTGTGCAGCAGCCGGCTCCGC
Reverse primer for A2 ΔCAR	GCCGACGCGTGTTAGCTGTGCAGCAGCCGGCTCCGC

Table 3-1 | **List of PCR primers for sub-cloning of the A2 CAR gene and their respective sequences.** *Colours denote specific sequences as follows:* GGATCC *= BamHI restriction site,* ACGCGT *= MluI restriction site,* ACCGCC *= Kozak consensus sequence,* TTA *= TAA stop codon,* G *= additional guanine nucleotide,* XYZXYZ *= complementary to A2 CAR gene sequence.*

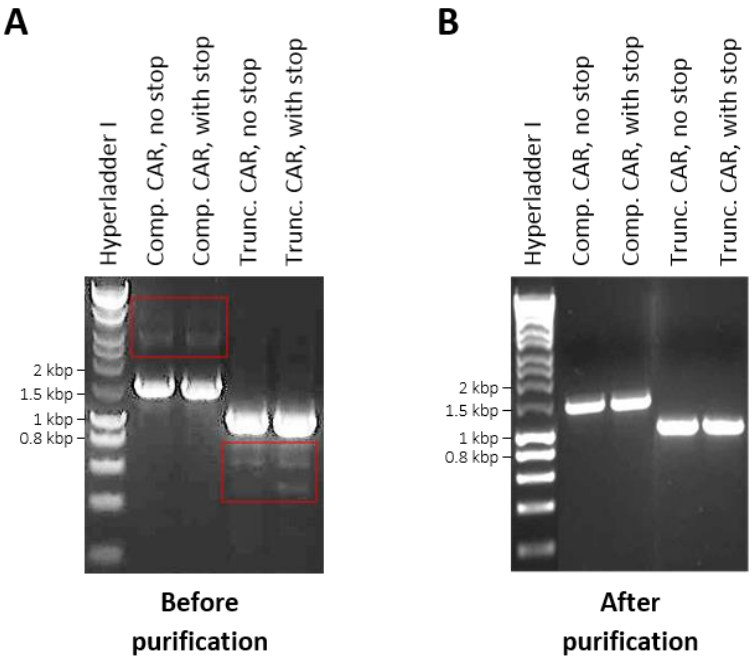

Figure 3-13 | **Purification of CAR gene PCR products using a membrane-based purification system.** *CAR gene PCR products were purified using the membrane-based Wizard® SV Gel and PCR Clean-Up System. PCR products before (A) and after (B) purification are shown on a 0.8% agarose gel alongside the Hyperladder I. Contaminants are highlighted with red boxes.*

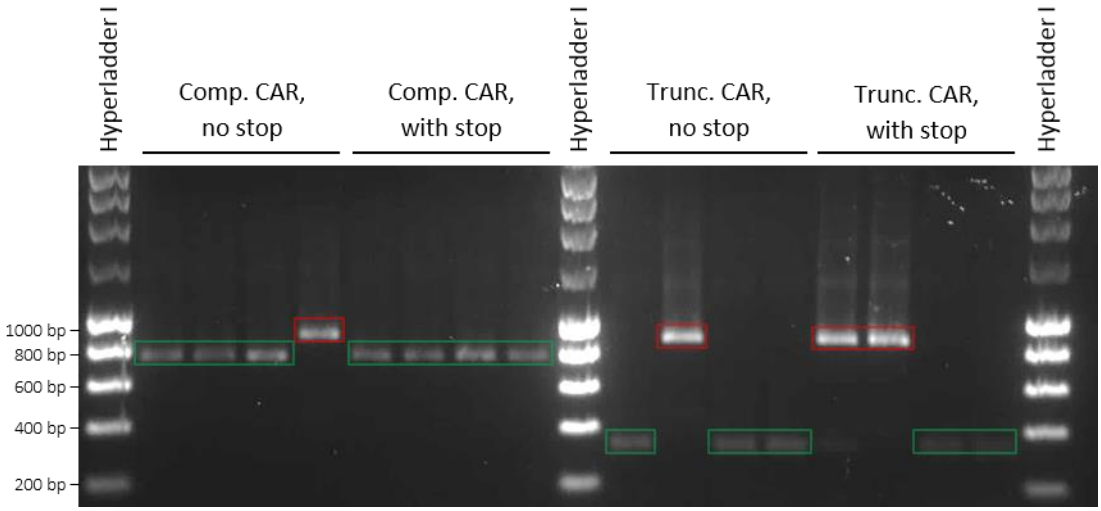

Figure 3-14 | **NotI restriction digest of pCR™ vector containing A2 CAR gene inserts**. *CAR gene inserts generated from a PCR amplification process were blunt-end ligated into the pCR™ vector. DNA ligation products were expanded, purified by miniprep and restriction digested with NotI. Digest fragments were separated on a 0.8% agarose gel alongside the Hyperladder I. Correctly orientated CAR gene inserts were identified by bands of 4,300 and 720 bp (complete (comp.) CAR) or 4,300 and 300 bp (truncated (trunc.) CAR), as highlighted with green boxes. Conversely, incorrectly orientated CAR gene inserts resulted in fragments of 4,100 and 865 bp (comp. CAR) or 3,700 and 900 bp (trunc. CAR), highlighted with red boxes.*

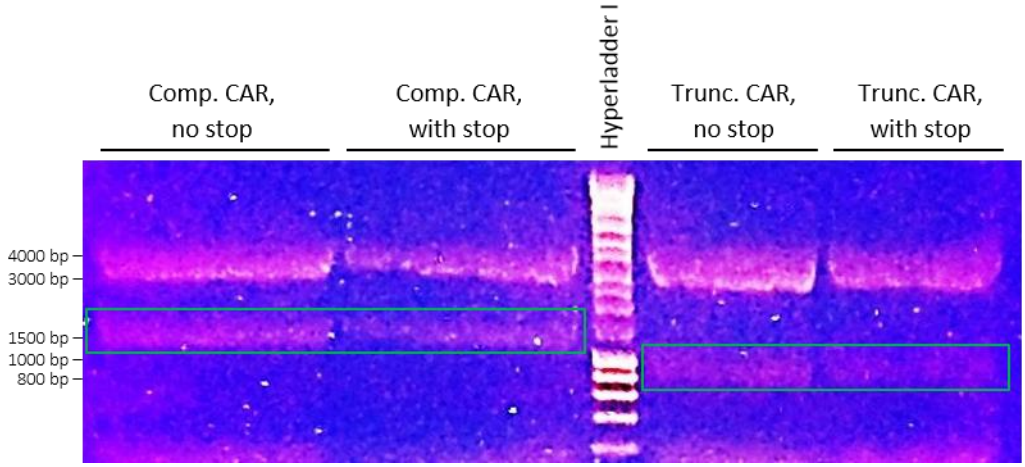

Figure 3-15 | **BamHI and MluI restriction digest of pCR™ vectors containing the A2 CAR gene inserts.** *pCR™ vectors containing the A2 CAR gene inserts in the correct orientation were restriction digested with BamHI and MluI. CAR gene inserts with 5' BamHI and 3' MluI sticky ends were separated on a 0.6% agarose gel and visualised using a long wavelength ultraviolet transilluminator to minimise photo-nicking damage (green boxes). These bands were cut out and DNA was extracted using the membrane-based Wizard® SV Gel and PCR Clean-Up System.*

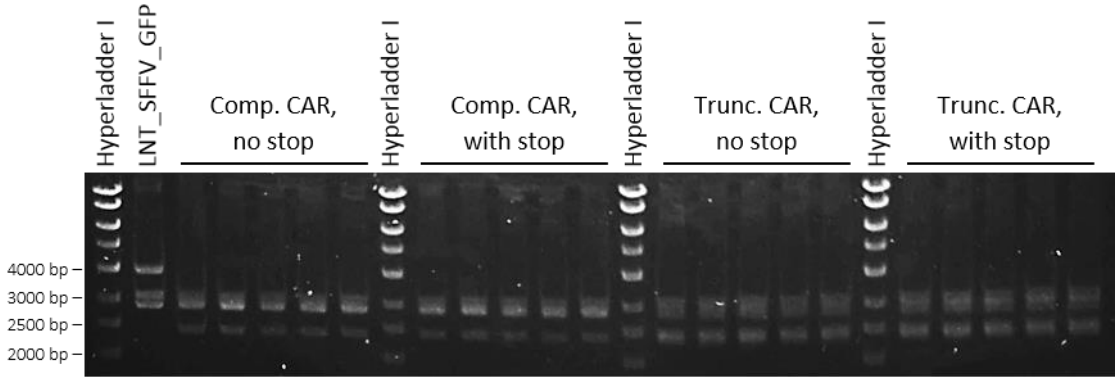

Figure 3-16 | **PvuII restriction digest of pLNT/SFFV_eGFP vectors containing A2 CAR gene inserts.** *A2 CAR gene inserts were ligated into the target pLNT/SFFV_eGFP vector. DNA ligation products were expanded, purified by miniprep and restriction digested with PvuII. Digest fragments were separated on a 0.8% agarose gel alongside the Hyperladder I. pLNT/SFFV_eGFP vectors containing a CAR insert yielded predicted PvuII digest fragments of 3,100, 2,800 and 2,400 bp.*

BamHI and MluI sticky ends. DNA was separated by agarose gel electrophoresis and bands of 1,494 bp (complete CAR) and 1,063 (truncated CAR) were extracted and purified using a membrane-based purification system (Figure 3-15, extracted bands highlighted with green boxes). Complete and truncated CAR DNA inserts were ligated into the pLNT/SFFV_eGFP vector using a T4 ligase. Stbl3 cells were then transformed with the ligation products and grown on agar plates in the presence of ampicillin to selectively expand successfully transformed *E. coli*. DNA from single colonies was isolated by miniprep and correctly ligated vectors were identified by PvuII restriction digest. DNA bands of 3,100, 2,800 and 2,400 bp were expected for vectors containing the full-length or truncated CAR genes (Figure 3-16) and were observed in all cases. Subsequent sequencing analysis confirmed that this cloning process had successfully been performed without the incorporation of a point mutation with the A2-28ζ-eGFP ORF.

3.3.3.1 The full-length A2-28ζ CAR remains functional with a C-terminal eGFP fusion

During the sub-cloning process, the C-terminus of the A2 CAR was modified, either by the addition of an eGFP fusion or the removal of the CD28-CD3ζ signalling domain. To evaluate the potential consequences of these modifications in terms of their functionality, the aforementioned A2 CARs were assessed by measuring their ability to activate T cells in the presence of HLA-A2. Hereafter, the full-length CARs with and without the eGFP fusions were termed A2 CAR-eGFP and A2 CAR,

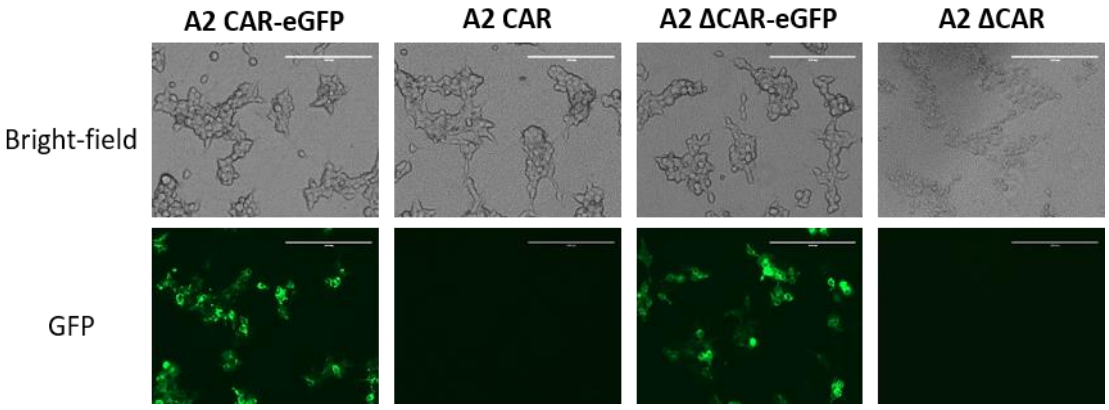

Figure 3-17 | **Fluorescence microscopy images of HEK293T cells transfected to express the pLNT/SFFV_CAR-eGFP constructs.** *HEK293T cells were co-transfected to express a pLNT/SFFV_CAR-eGFP plasmid, a pCMV-ΔR8.91 packaging plasmid and a pCMV-VSV-G envelope plasmid, resulting in the production of VSV-G-pseudotyped lentiviral particles. HEK293T cells were visualised by fluorescence microscopy 24 hours post-transfection. Images were taken at x20 magnification with a scale bar which represents 200 µm. Data representative of 3 individual experiments.*

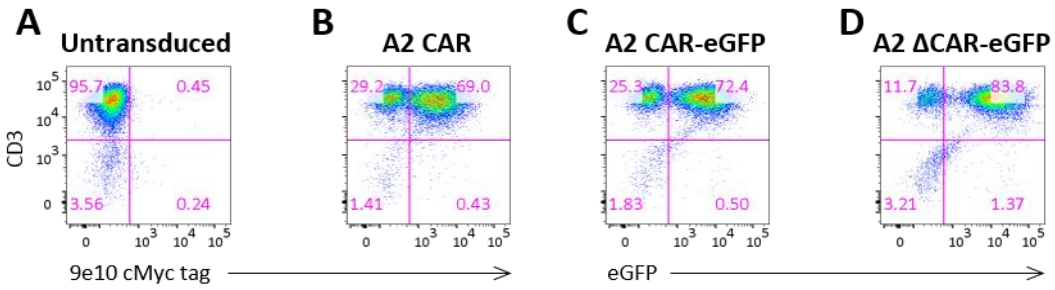

Figure 3-18 | **Unfractioned human T cells were transduced to express CAR genes with a high efficiency using VSV-G-pseudotyped lentiviral particles.** *Human PBMCs were isolated from a HLA-A2⁻ donor and activated with PHA-L and IL-2. Activated T cells were left untransduced (A) or lentivirally transduced 72 hours post-activation with VSV-G-pseudotyped lentiviral particles to express the A2 CAR (B), the A2 CAR with a C-terminal eGFP fusion (A2 CAR-eGFP, C) or the truncated A2 CAR with a C-terminal eGFP fusion (A2 ΔCAR-eGFP, D). The transduction efficiency was measured 7 days post-transduction by flow cytometry, using antibodies specific for the 9E10 cMyc epitope (B) or eGFP expression (C/D). Data representative of 2 individual experiments.*

respectively whilst the truncated CARs which lacked a CD28-CD3ζ signalling domain were termed A2 ΔCAR-eGFP and A2 ΔCAR, respectively.

VSV-G-pseudotyped lentiviral particles containing the various A2 CAR constructs were generated by co-transfecting HEK293T cells with a corresponding pLNT/SFFV_CAR-eGFP plasmid, a pCMV-ΔR8.91 packaging plasmid and a pCMV-VSV-G envelope plasmid. The transfection of these HEK293T cells was confirmed by assessing eGFP expression using fluorescence microscopy (Figure 3-17). This indicated that the A2 CAR-eGFP and ΔCAR-eGFP fusion proteins were successfully expressed. Furthermore, eGFP was not detected in the HEK293T cells which were transfected with the A2 CAR and ΔCAR. As the eGFP ORF was still present in these CAR constructs, this suggested that there was no "leaky" eGFP expression in this system.

Viral supernatant was harvested 48-56 hours post-transfection and VSV-G-pseudotyped lentiviral particles were concentrated using a precipitation approach. HLA-A2⁻ PBMCs were activated with PHA and IL-2, as previously described (section 3.3.2.1), and transduced to express 1 of the 4 A2 CAR constructs after 72 hours. The transduction efficiency of the T cells was assessed by flow cytometry 7 days post-transduction (Figure 3-18) where CAR expression was quantified either by measuring eGFP expression for CARs which were fused to eGFP (Figure 3-18C/D), or staining with an anti-9E10 cMyc epitope antibody for CARs which lacked an eGFP fusion (Figure 3-18B). $CD3^+$ T cells were identified using an anti-human CD3 antibody. Using this lentiviral approach, a transduction efficiency of 62.4 ± 28.0% was achieved (n=4) which was a vast improvement over

the 9.9 ± 7.6% that was achieved for the retroviral approach (n=4; Figure 3-5A). It was therefore concluded a lentiviral CAR delivery approach was more appropriate for downstream experiments.

To confirm the functionality of the CARs, A2 CAR-eGFP, CAR and ΔCAR-eGFP T cells were co-cultured with confluent monolayers of MCF-7 and T-47D cells, as previously described (section 3.3.2.2). As expected, A2 CAR T cells exhibited cytotoxicity towards HLA-A2$^+$ MCF-7 cells (Figure 3-19A) and similar findings were observed for A2 CAR-eGFP T cells. In contrast, A2 ΔCAR-eGFP T cells did not exhibit cytotoxicity. No significant difference was measured in the

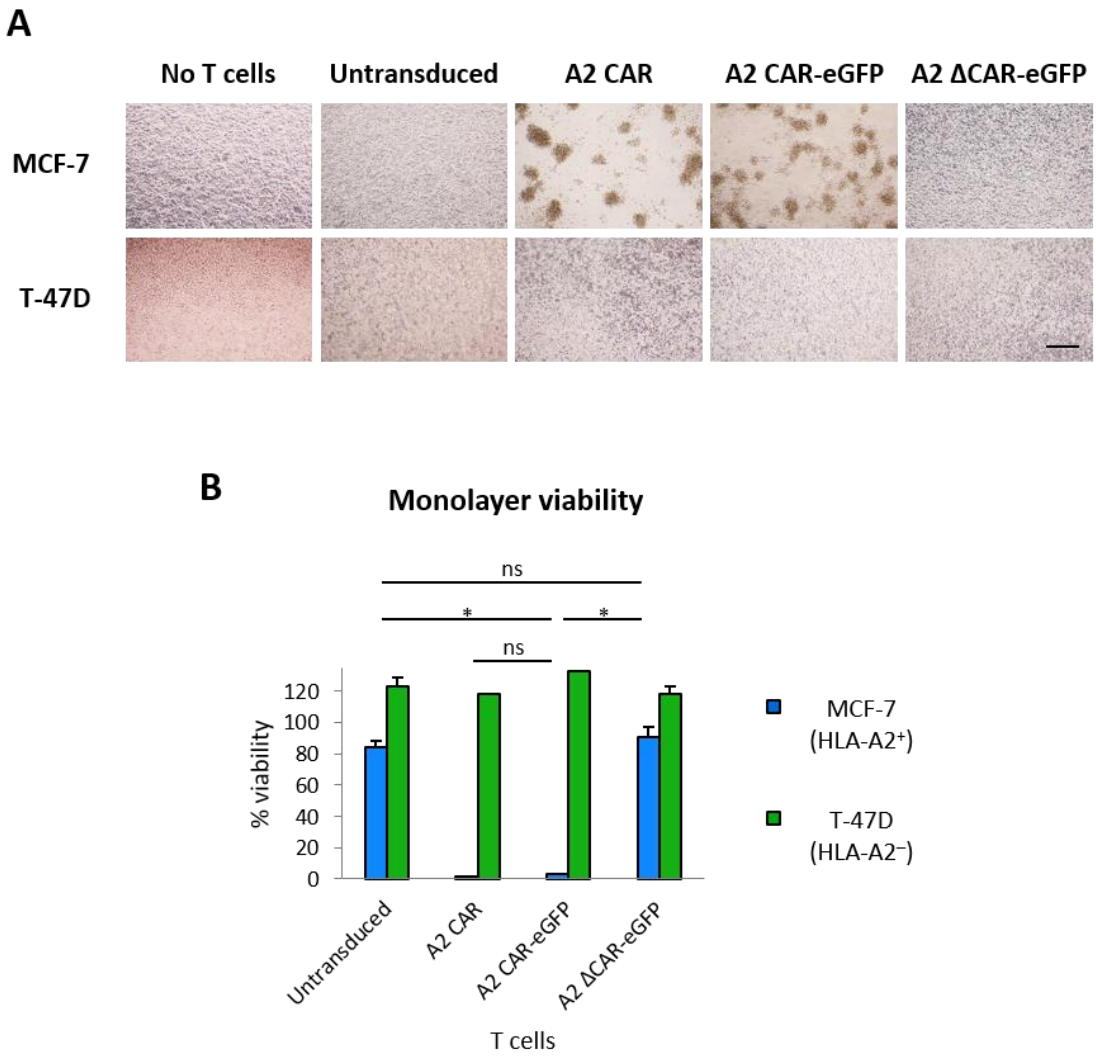

Figure 3-19 | **T cells expressing the A2 CAR-eGFP fusion exhibited cytotoxicity with a similar potency to T cells expressing the A2 CAR.** *A: CAR-modified T cells were co-cultured overnight with confluent monolayers of MCF-7 and T-47D cells. Images were taken after the co-culture at x10 magnification. Scale bar = 250 µm. B: The viability of the cell monolayers following co-culture was quantified by MTT assay. % viability of MCF-7 (blue bars) and T-47D (green bars) cells is shown relative to monolayer cells cultured alone. Data is mean ± SD and is representative of 2 individual experiments. Significance was determined using a two-tailed paired Student's t-test where *=p<0.05.*

ability of A2 CAR and A2 CAR-eGFP T cells to kill HLA-A2$^+$ MCF-7 cells (MCF-7 viability with A2 CAR T cells = 2.8 ± 0.3% and with A2 CAR-eGFP T cells = 1.8 ± 0.1%, p=0.083; representative of 2 individual experiments; Figure 3-19B). Furthermore, presence of the A2 ΔCAR-eGFP construct in T cells did not cause an increase in cytotoxic activity elicited towards HLA-A2$^+$ MCF-7 cells, relative to untransduced T cells (MCF-7 viability with untransduced T cells = 84.3 ± 3.5% and with A2 ΔCAR-eGFP T cells = 90.9 ± 5.8%, p=0.16). As this A2 ΔCAR-eGFP construct comprised the same HLA-A2-targeting moiety as the full-length A2 CARs, this finding suggested that the CARs must possess a CD28-CD3ζ signalling domain in order to elicit cytotoxic activity. Overall, these results demonstrated that the presence of the eGFP fusion did not influence the functionality of the A2 CAR. In contrast, removal of the CD28-CD3ζ signalling domain severely compromised the functionality of the CAR.

Analysis of the cytokines produced during the co-cultures complemented these findings. T cells expressing the A2 CAR and A2 CAR-eGFP constructs produced similar levels of IL-2 (11.9 ± 0.34 ng/mL and 13.3 ± 0.27 ng/mL; Figure 3-20A) and IFNγ (28.6 ± 1.2 ng/mL and 34.7 ± 0.18 ng/mL; representative of 3 individual experiments; Figure 3-20B) in the presence of HLA-A2$^+$ MCF-7 cells with no significant differences in the quantities secreted (IL-2 p=0.49; IFNγ p=0.074).

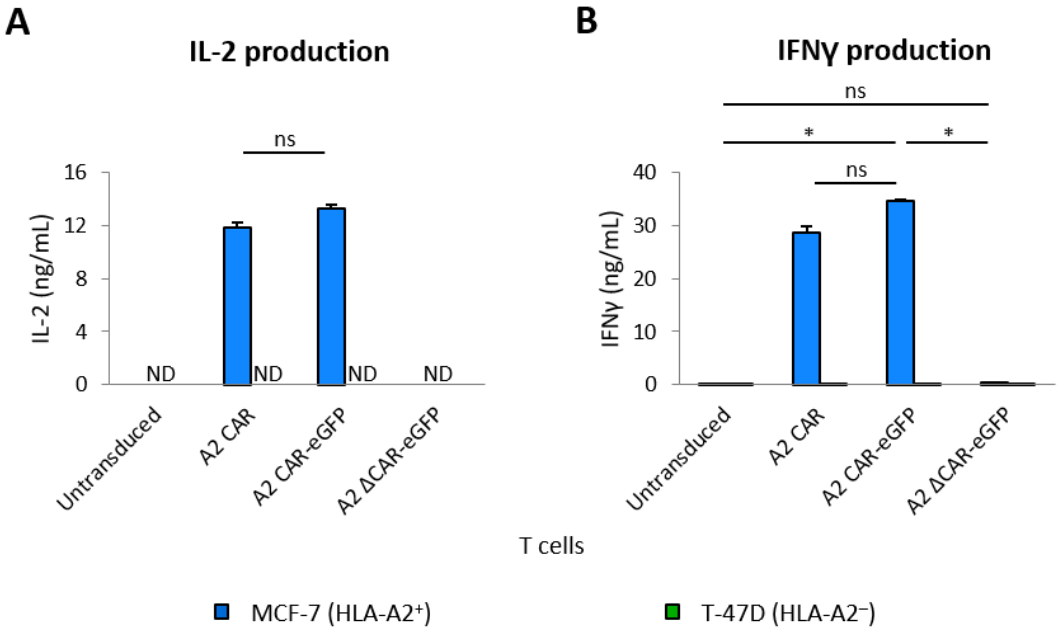

Figure 3-20 | **T cells expressing the A2 CAR and A2 CAR-eGFP produce similarly high levels of IL-2 and IFNγ in the presence of HLA-A2$^+$ target cells.** *Supernatants from co-cultures of CAR-modified T cells and MCF-7 (blue) and T-47D (green) cells were analysed for production of IL-2 (A) and IFNγ (B) by cytokine-specific ELISAs. Data is mean ± SD and is representative of 3 individual experiments. Significance was determined using a two-tailed paired Student's t-test where *=p<0.05 and **=p<0.01.*

No detectable levels of IL-2 were produced in any of the other conditions analysed. The quantity of IFNγ produced by the A2 CAR and A2 CAR-eGFP T cells was >100-fold greater than untransduced (212.5 ± 7.3 pg/mL; p<0.019) and A2 ΔCAR-eGFP (252.8 ± 19.6 pg/mL; p<0.019) T cells. Overall, these results demonstrated that the presence of a C-terminal eGFP fusion did not influence the functionality of the A2 CAR. Additionally, removal of the CD28-CD3ζ signalling domain from this A2 CAR resulted in the generation of a non-functional HLA-A2-specific CAR which was capable of recognising HLA-A2 but was not able to facilitate T cell activation.

3.3.3.2 The cytotoxic activity of A2 CAR-eGFP Tconvs is not influenced by the presence of a commercial HLA-A2-specific blocking antibody

To further characterise the specificity of the A2 CAR, the previously described cytotoxicity assay was performed in the presence and absence of a HLA-A2-specific blocking antibody. Over the past 40 years, a variety of HLA-A2-specific monoclonal antibodies have been developed. However, the use of the majority of these clones has been superseded by the BB7.2 clone to the extent that, to our knowledge, this is currently the only HLA-A2-specific antibody clone which is commercially available. Confluent monolayers of HLA-A2$^+$ MCF-7 cells were cultured with 20 µg/mL of the BB7.2 antibody or an IgG2b control for 30 minutes before being co-cultured with 1x10^6 CD4$^+$CD25$^-$ Tconvs which were untransduced or engineered to express the A2 CAR-eGFP or A2 ΔCAR-eGFP construct. Co-cultures were performed overnight in the presence of 10 µg/mL antibody. The viability of the MCF-7 cells was then measured by MTT assay.

A2 CAR-eGFP Tconvs co-cultured in the presence of the BB7.2 antibody exhibited a similar level of cytotoxicity to A2 CAR-eGFP Tconvs which were cultured in the absence of an antibody or presence of the IgG2b control antibody (Figure 3-21A). This suggested that recognition of HLA-A2 by the A2 CAR was not blocked by the presence of the BB7.2 CAR. This may be explained by the locations of the predicted epitopes for the A2 CAR scFv and the BB7.2 antibody, as shown in Figure 3-21B.

3.4 DISCUSSION

In this chapter, a second generation CAR comprising a human-derived HLA-A2-targeting moiety and CD28-CD3ζ signalling domain was designed and generated. This A2 CAR was cloned into an

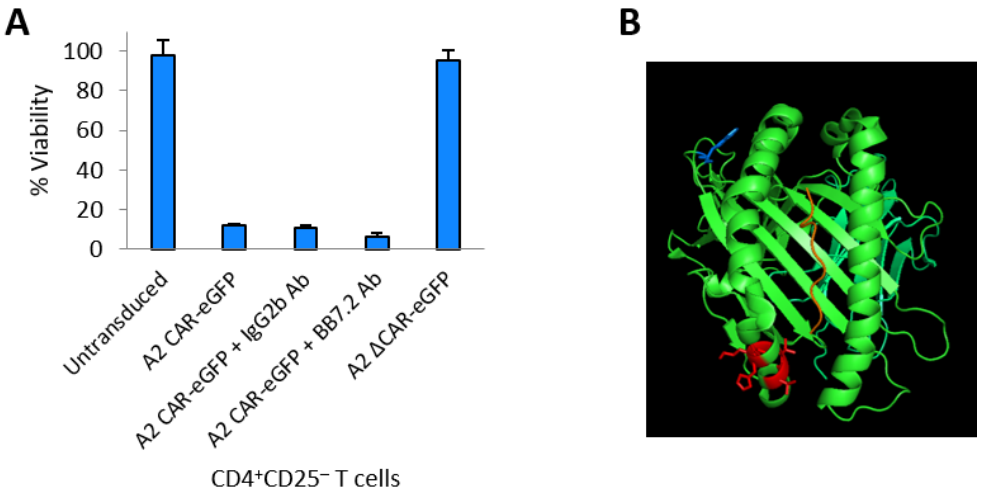

Figure 3-21 | **The cytotoxic activity of A2 CAR-eGFP Tconvs is not blocked by the presence of a HLA-A2-specific blocking antibody.** *A: $CD4^+CD25^-$ Tconvs expressing the A2 CAR-eGFP construct were co-cultured overnight with confluent monolayers of HLA-$A2^+$ MCF-7 cells in the presence of 10 µg/mL of an anti-HLA-A2 monoclonal antibody (BB7.2 clone). The viability of the MCF-7 cells was then measured by MTT assay. Data represents mean ± SD where % viability is shown relative to monolayer cells. B: Molecular graphic of HLA-A2 (PDB: 2AV1) with the predicted A2 CAR scFv epitope (Watkins, Brown et al. 2000) highlighted in red (amino acids 142-145 in the α2 chain) and the predicted epitope of the BB7.2 antibody clone (amino acids 107 in the α2 chain) highlighted in blue. Image generated using PyMOL software (Schrödinger LLC, New York, USA).*

SFG retroviral vector and delivered into HLA-$A2^-$ T cells with an efficiency of approximately 10%. When A2 CAR T cells were co-cultured HLA-$A2^+$ MCF-7 cell monolayers, T cell activation was observed whereby cytotoxicity was elicited and pro-inflammatory cytokines were produced, suggesting that the A2 CAR was functional and specific for HLA-A2. In an aim to increase the efficiency with which target T cells were transduced, the A2 CAR gene was sub-cloned into a second generation LNT/SFFV_eGFP lentiviral vector. During this process, an eGFP C-terminal fusion was added to the A2 CAR and a second CAR was generated to comprise the same HLA-A2-targeting moiety without a CD28-CD3ζ signalling domain (A2 ΔCAR-eGFP). Employing a lentiviral transduction approach enabled the A2 CAR to be delivered into PBMCs with an efficiency of approximately 60%. Presence of a C-terminal eGFP fusion did not influence the functionality of the A2 CAR. However, removal of the CD28-CD3ζ signalling domain was shown to severely compromise the ability of this CAR to activate T cells, despite the fact that this CAR was still able to engage HLA-A2.

The expression of mouse-derived sequences in human cells has the potential to trigger undesired immune responses *in vivo*. This is particularly true for proteins which have elements exposed on

the cell surface, such as the scFv of a CAR. The severity of this issue has been demonstrated in two clinical trials in which CAR T cells containing a murine-derived scFv did not persist long-term post-transfer (Kershaw, Westwood et al. 2006, Lamers, Willemsen et al. 2011). Upon further investigation, this limited CAR T cell survival was attributed to the *in vivo* generation of antibodies which specifically recognised the CARs. The scFv employed to generate the A2 CAR in this project was of human origin (Watkins, Brown et al. 2000). This reduced the immunogenicity of the CAR and improved the chances of CAR Tregs surviving long-term post-transfer. In its original description, this HLA-A2-specific scFv sequence was shown to cross-react with HLA-A28 and HLA-A68. This cross-reactivity has not been confirmed using the A2 CAR but would be advantageous in a clinical setting, provided that the recipients lacked expression of all three of these HLA class I antigens. In a cancer setting, a CAR specific for one antigen can be used in the treatment of many patients. However, in the envisaged transplant setting, it would be necessary to generate a library of numerous CARs targeting different HLA class I molecules to accommodate the allele variability between different donors and recipients. Using cross-reactive CARs which target multiple HLA class I molecules would significantly reduce the number of CARs which would need to be developed to adopt CAR Tregs in the clinic.

Recently, a HLA-A2-specific CAR was generated by MacDonald *et al*. who demonstrated the ability of CAR Tregs to inhibit xeno-GvHD (MacDonald, Hoeppli et al. 2016). These authors used a hybridoma-derived scFv sequence from the BB7.2 anti-HLA-A2 antibody clone (section 3.3.3.2) to comprise their antigen-targeting moiety. However, the elements downstream of this scFv including the cMyc tag, the CD28 transmembrane domain and the CD28-CD3ζ signalling domain were identical to the CAR generated in this chapter. This CAR was also delivered by lentiviral transduction into target cells and was shown to effectively confer HLA-A2-specificity onto Tconvs. This was demonstrated by stimulating A2 CAR-expressing $CD4^+CD25^-$ T cells with $HLA\text{-}A2^+$ stimulator cells and measuring the upregulation of several activation markers and the production of various cytokines. These findings were similar to the results presented in this chapter.

In cancer research, control CARs are typically generated by mutating either the ectodomain (Kuramitsu, Ohno et al. 2015, Whilding, Parente-Pereira et al. 2016) or the endodomain (Bridgeman, Ladell et al. 2014, Kobold, Grassmann et al. 2015) of a functional CAR of interest. Mutating the endodomain is typically preferred as this allows the T cells to engage their target antigen and preferentially migrate to where this antigen is expressed *in vivo*. As such, a HLA-A2-specific CAR lacking a CD28-CD3ζ signalling domain was generated to investigate whether preferential Treg homing to the allograft was sufficient to elicit protection from graft rejection. The idea of using a mutated CAR has not previously been performed for CAR Tregs.

3.4.1 SUMMARY

Overall, the results presented in this chapter demonstrated the specificity and functionality of a novel HLA-A2-specific CAR. This CAR was efficiency delivered into human T cells through lentiviral transduction. The following chapter will explore whether lentiviral delivery of this CAR into human Tregs influences the nature of these cells *in vitro*.

CHAPTER IV

GENERATION OF HUMAN REGULATORY T CELLS EXPRESSING A HLA-A2-SPECIFIC CHIMERIC ANTIGEN RECEPTOR

4.1 INTRODUCTION

4.1.1 TREG ISOLATION FOR CLINICAL USE

Clinical-grade human Tregs can be isolated by using one of two GMP-compatible approaches: i) to FACS sort Tregs using a range of different cell surface markers such as CD4, CD25 and CD127, or; ii) to enrich $CD8^{-}CD25^{+}$ T cells using sequential bead-based negative and positive selection protocols.

Cell sorting is advantageous as it allows Tregs to be isolated with a high purity. Consequently, this approach is preferred and currently used for clinical trials outside of the UK. However, current GMP guidelines restrict the use of this approach in the UK, due in part to the lack of a suitable "closed system" cell sorter. This will likely be resolved in the near future with the development of the MACSQuant® Tyto™ cell sorter (Miltenyi Biotec). However, this has impacted previous and ongoing UK-based trials which have been restricted to enriching $CD8^{-}CD25^{+}$ Tregs using paramagnetic bead-based negative and positive selection protocols in combination with a CliniMACS closed system (Miltenyi Biotec) which inevitably yields a less pure population of Tregs.

4.1.2 TREG EXPANSION FOR CLINICAL USE

In transplant-based clinical trials, Tregs are isolated under GMP-compatible conditions and expanded *ex vivo* to yield a therapeutically applicable number of cells. This can be achieved by activating the Tregs using one of two approaches, allowing cells to be expanded in a polyclonal or antigen-specific manner. For the former, the Tregs are activated with GMP-compatible anti-human CD3/CD28 beads. This approach is currently used for generating the cell products which are used in The ONE Study and ThRIL trials based at Guy's Hospital. Alternatively, Tregs with direct allospecificity can be preferentially expanded by culturing the cells with allogeneic donor-derived APCs which can be in the form of PBMCs (Peters, Hilbrands et al. 2008, Jeffery, Braitch et al. 2016), DCs (Sagoo, Ali et al. 2011, Cherai, Hamel et al. 2015) or B cells (Tu, Lau et al. 2008, Chen, Delgado et al. 2009, Zheng, Liu et al. 2010, Noyan, Lee et al. 2013, Putnam, Safinia et al. 2013).

Following activation, the Tregs are expanded *ex vivo* in the presence of high levels of IL-2 (>500 U/mL) although the period of time for which the Tregs are expanded varies between trials. For example, the original standard operating procedures (SOP) for The ONE Study and ThRIL at King's College London dictated that the Tregs had to be expanded for 36 days where fresh anti-CD3/CD28 beads were provided on days 12 and 24 to re-stimulate the cells. This has since

been modified such that if a sufficient number of cells is acquired after 2 simulation procedures (24 days), the cells can be cryopreserved at this point in their expansion protocol. Conversely, Bluestone *et al*. recently published data from a clinical trial where autologous Tregs were expanded for 14 days with just one stimulation procedure before being transferred back into T1D patients (Bluestone, Buckner et al. 2015). Despite this shorter culture period, $CD4^{+}CD25^{+}CD127^{lo}$ Tregs in this trial were efficiently expanded with an average fold-increase of 554.7 ± 370.2.

4.1.2.1 Tregs and rapamycin

The translation of Treg therapy into the clinic was hindered for many years due to concerns regarding the purity of the Tregs, particularly as the only GMP-compatible approach which was available during this time was to enrich Tregs by negative and positive selection. It was realised that expanding Tregs in the presence of high levels of IL-2 alone allowed for the concurrent expansion of contaminating T cells which could potentially promote graft rejection if adoptively transferred. Secondary concerns were also raised regarding the stability of the expanded Tregs following transfer. This was based on studies which had demonstrated that Tregs were susceptible to converting into Th17 cells when in a pro-inflammatory environment (Koenen, Smeets et al. 2008, Yang, Nurieva et al. 2008, Scotta, Esposito et al. 2013), such as that which would be expected in an allograft which is undergoing rejection. Again, in this scenario, transferred cells would promote graft rejection.

These issues were subdued by various studies which demonstrated that expanding T cells in the presence of rapamycin both allowed for the selective expansion of Tregs and enhanced the stability of these cells (Battaglia, Stabilini et al. 2005, Strauss, Whiteside et al. 2007, Basu, Golovina et al. 2008, Golovina, Mikheeva et al. 2011, Scotta, Esposito et al. 2013). Rapamycin is one of the immunosuppressive drugs used *in vivo* to reduce solid organ transplant rejection (section 1.1.2.1) (Touzot, Soulillou et al. 2012). However, the ability of rapamycin to selectively promote the expansion of Treg makes it a useful asset for growing Tregs *ex vivo* thus it commonly features in GMP-compatible Treg expansion protocols where it is usually supplemented into culture media at a concentration of 100 nM. Presence of exogenous rapamycin in *ex vivo* T cell cultures favours the expansion of Tregs, allowing these cells to outgrow contaminating Tconvs which may exist in an isolated Treg preparation, particularly when these cells have been enriched using a bead-based negative and positive selection protocol. Similar preferential expansion of Tregs in the presence of rapamycin has also been observed in mouse models (Hester, Schiopu et al. 2012) and suggested in renal and liver transplant recipients (Segundo, Ruiz et al. 2006, Noris, Casiraghi et al. 2007, Akimova, Kamath et al. 2012).

The principle target or rapamycin is mTOR, a serine/threonine kinase which plays an important role in many cell processes including cell adhesion, growth, proliferation and survival (Ballou and Lin 2008). mTOR regulates these processes as a component of a protein complex. These protein complexes exist in different forms and are defined by the additional proteins included in the complex. By combining with regulatory-associated protein of mTOR (Raptor), mTOR complex 1 (mTORC1) is formed whilst combining with rapamycin-insensitive companion of mTOR (Rictor) results in the formation of mTOR complex 2 (mTORC2). mTORC1 acts downstream of AKT and phosphorylates numerous target proteins to influence a wide variety of cell processes. In contrast, mTOCR2 is currently believed to act solely on AKT (Wang, Camirand et al. 2011). Although the mechanisms are not fully understood, it is widely reported that rapamycin primarily functions by inhibiting mTORC1 (Shimobayashi and Hall 2014). However, studies have suggested that rapamycin may also influence mTORC2 (Sarbassov, Ali et al. 2006, Yin, Hua et al. 2016).

IL-2 signalling, which is of fundamental importance for T cell survival (section 1.1.3.2.1), is transmitted intracellularly through two signalling pathways: i) the phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR pathway, and; ii) the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Safinia, Scotta et al. 2015). Studies have demonstrated that proliferating Tregs are less dependent than Tconvs on signalling through the PI3K-AKT-mTOR pathway and instead, Tregs preferentially rely on signalling through STAT5 (Bensinger, Walsh et al. 2004, Zeiser, Leveson-Gower et al. 2008), suggesting a mechanism by which rapamycin promotes Treg expansion.

4.1.3 Genetic manipulation of human Tregs

Although gene-modified Tregs do not feature heavily in clinical trials, various studies have demonstrated that genetic manipulation of human Tregs is possible. This has typically been performed to generate antigen-specific Tregs by transducing these cells to express specific TCR α and β chain genes (Brusko, Koya et al. 2010, Plesa, Zheng et al. 2012, Kim, Zhang et al. 2015) or CARs (Hombach, Kofler et al. 2009, MacDonald, Hoeppli et al. 2016). In these studies, there was a preference to deliver the GOIs using a lentiviral approach rather than a retroviral approach. Furthermore, transduced cells were shown to maintain both their phenotype and function in a polyclonal manner, whilst exhibiting a superior suppressive capacity in an antigen-specific manner.

4.2 AIMS AND OBJECTIVES

The aim of this chapter was to generate human Tregs which expressed either the A2 CAR-eGFP or the A2 ΔCAR-eGFP construct. To enhance the translatability of these findings, the Tregs were isolated using protocols which are relatable to those currently employed in the GMP facility at Guy's Hospital, i.e. $CD4^+CD25^+$ Tregs were enriched using sequential negative and positive selection protocols. A protocol was then optimised to efficiency deliver the A2 CAR-eGFP or A2 ΔCAR-eGFP genes into these Tregs, without influencing the phenotype or suppressive ability of these cells, as assessed by flow cytometry and investigating the ability of the Tregs to suppress autologous $CD4^+CD25^-$ Tresp proliferation *in vitro*, respectively.

4.3 RESULTS

4.3.1 ISOLATION, EXPANSION AND CHARACTERISATION OF HUMAN TREGS EXPANDED IN THE PRESENCE OF RAPAMYCIN

4.3.1.1 $CD4^+CD25^+$ human Tregs were enriched from peripheral blood using a GMP-compatible protocol with a high purity

Human $CD4^+CD25^+$ Tregs were enriched from the peripheral blood of healthy donors using a previously established GMP-compatible protocol (Afzali, Edozie et al. 2013, Scotta, Esposito et al. 2013, Safinia, Vaikunthanathan et al. 2016). $CD4^+$ PBMCs were enriched by negative selection, specifically depleting cells which expressed CD8, CD16, CD19, CD36, CD56, CD66b and γ/δ TCRs. This was followed by a positive selection step in which $CD25^+$ cells were enriched. Using this protocol, a $CD4^+CD25^+$ purity of approximately 90% (89.5 ± 4.4%, n=16) was achieved (Figure 4-1A). A detailed phenotypic analysis of these cells (Figure 4-1B/C) demonstrated that a high proportion (79.0 ± 14.9%, n=6) of the cells expressed the lineage-determining Treg transcription factor FOXP3 (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Fontenot, Rasmussen et al. 2005) whilst a low proportion (30.1 ± 9.1%) expressed the IL-7 receptor α chain, CD127 (Liu, Putnam et al. 2006). CTLA-4 (46.0 ± 14.4%) and CD39 (55.1 ± 12.0%) were not expressed in all of the isolated cells, in line with previous studies which have demonstrated that whilst the majority of $CD4^+CD25^+$ human T cells express these markers, not *all* of these cells do (Dieckmann, Plottner et al. 2001, Jago, Yates et al. 2004, Borsellino, Kleinewietfeld et al. 2007). This result may be

partially attributed to the presence of contaminating activated T cells which have downregulated CTLA-4 or CD39 expression (Jago, Yates et al. 2004).

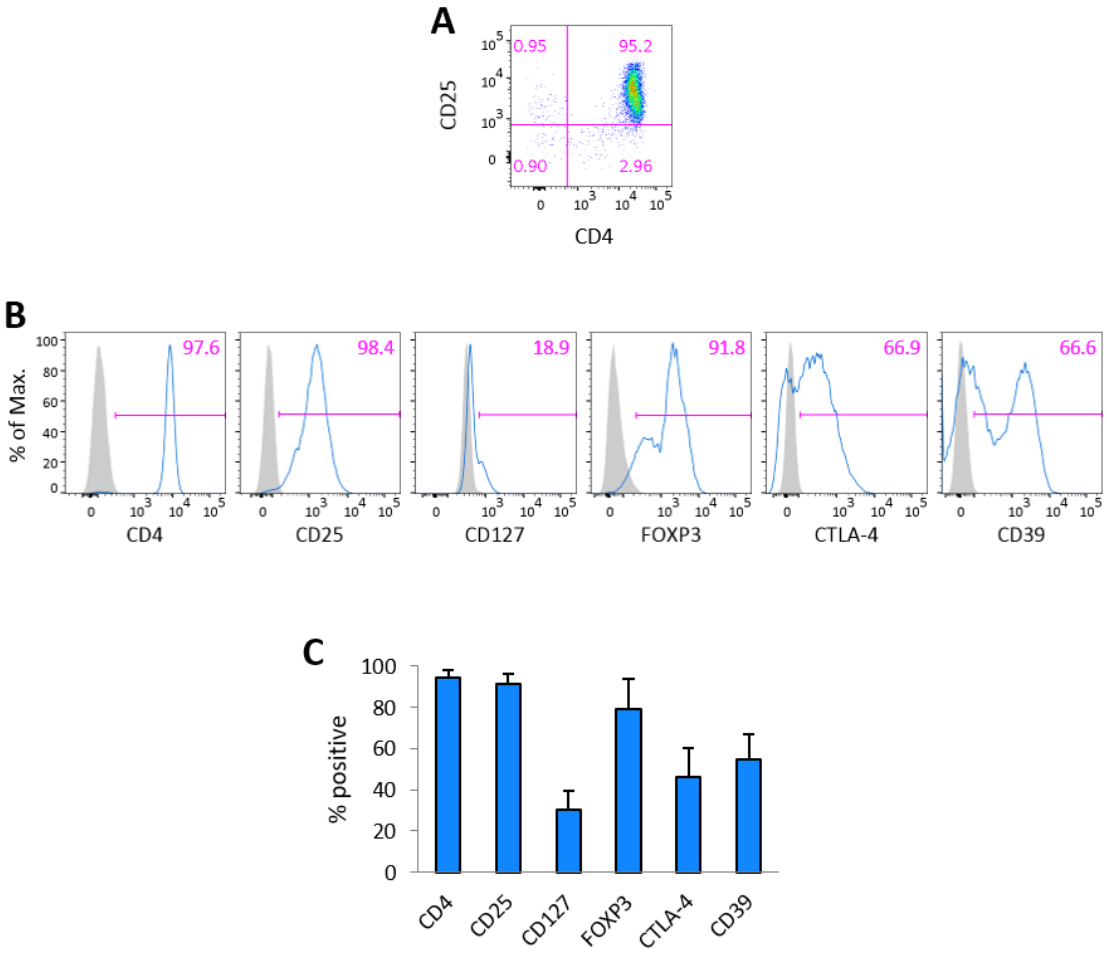

Figure 4-1 | **Purity and phenotype of CD4⁺CD25⁺ Tregs freshly isolated from the peripheral blood of healthy donors.** *CD4⁺ PBMCs were enriched by negative selection after which CD25⁺ cells were enriched by positive selection. The phenotype of these cells was assessed by flow cytometry. A: The purity of freshly isolated Tregs was determined by staining the cells with fluorescently-conjugated anti-human CD4 (OKT4 clone) and anti-human CD25 (4E3 clone) antibodies. Gate positions were defined on cells stained with isotype controls. Data representative of 16 individual experiments. B: An extensive phenotype of the freshly isolated Tregs was performed. Dead cells were excluded using a LIVE/DEAD dye and cells were stained with fluorescently-conjugated antibodies specific for CD4, CD25, CD127, FOXP3, CTLA-4 and CD39 (blue lines). Cells were fixed and permeabilised prior to staining of FOXP3 and CTLA-4. Cells stained with appropriate isotype controls are shown in solid grey and were used to define the gate positions shown. The percentage of stained cells within the gate shown is provided in the top left corner of each plot. Data representative of 6 individual experiments. C: Pooled data of the freshly isolated Treg phenotype. Data shown is mean + SD pooled from 6 individual experiments.*

4.3.1.2 Human Tregs cultured in the presence of rapamycin were highly suppressive

As discussed in the chapter introduction, culturing human $CD4^+CD25^+$ T cells in the presence of rapamycin has previously been demonstrated to selectively promote the expansion of $CD4^+CD25^+FOXP3^+$ Tregs (Battaglia, Stabilini et al. 2006, Scotta, Esposito et al. 2013). To assess whether these findings could be replicated using human Tregs which were isolated using the aforementioned Treg enrichment protocol, freshly isolated Tregs were expanded for 20 days in the presence or absence of 100 nM rapamycin (Figure 2-1). Following expansion, the phenotype and polyclonal suppressive capacity of the untreated and rapamycin-treated Tregs was compared.

Tregs cultured in the presence and absence of rapamycin exhibited a similar phenotype (Figure 4-2A). However, Tregs cultured in the presence of rapamycin expressed noticeably higher levels of Treg markers including FOXP3 (untreated MFI = 15,670; rapamycin-treated MFI = 20,293), CTLA-4 (untreated MFI = 1,783; rapamycin-treated MFI = 5,539) and CD39 (untreated MFI = 1,859; rapamycin-treated MFI = 2,257) although the overall proportion of cells expressing these markers was unaltered by the presence of this drug.

To investigate the suppressive capacity of the Tregs in a polyclonal manner, co-cultures of the Tregs and autologous $CD4^+CD25^-$ Tresps were performed to measure the ability of the Tregs to inhibit Tresp proliferation. Tresps were labelled with CTV, activated with a low-dose of anti-human CD3/CD28 beads (1:40 bead:Tresp ratio) and co-cultured with Tregs at Treg:Tresp ratios ranging from 1:1 to 1:32 for 5 days. Tresp proliferation was then analysed by flow cytometry, as determined by CTV dilution and the proliferation of these cells was calculated using division indices (FlowJo 7). Tresps cultured in the absence of Tregs typically achieved a division index of 0.95 ± 0.35 (n=6). Inhibition of Tresp proliferation was then used as a measure of suppression. As shown in Figure 4-2B, Tregs cultured in the presence of rapamycin were significantly more suppressive than Tregs cultured alone (*p*-values ranging from 0.0059 to $1.3x10^{-4}$; representative of 3 individual experiments). At a 1:1 ratio, untreated Tregs inhibited Tresps proliferation by 51.0% whilst rapamycin-treated Tregs suppressed 96.6% of the Tresp proliferation. Similarly, at a 1:16 Treg:Tresp ratio, no suppression was observed for untreated Tregs whilst the rapamycin-treated Tregs remained highly suppressive, inhibiting 61.3% of the Tresp proliferation, proving the potency of these cells.

Overall, expanding human $CD4^+CD25^+$ Tregs in the presence of rapamycin resulted in cells which expressed higher levels of known Treg markers including FOXP3 and the functional molecules

CTLA-4 and CD39. This phenotype may have contributed to the superior suppressive capacity these cells exhibited *in vitro*.

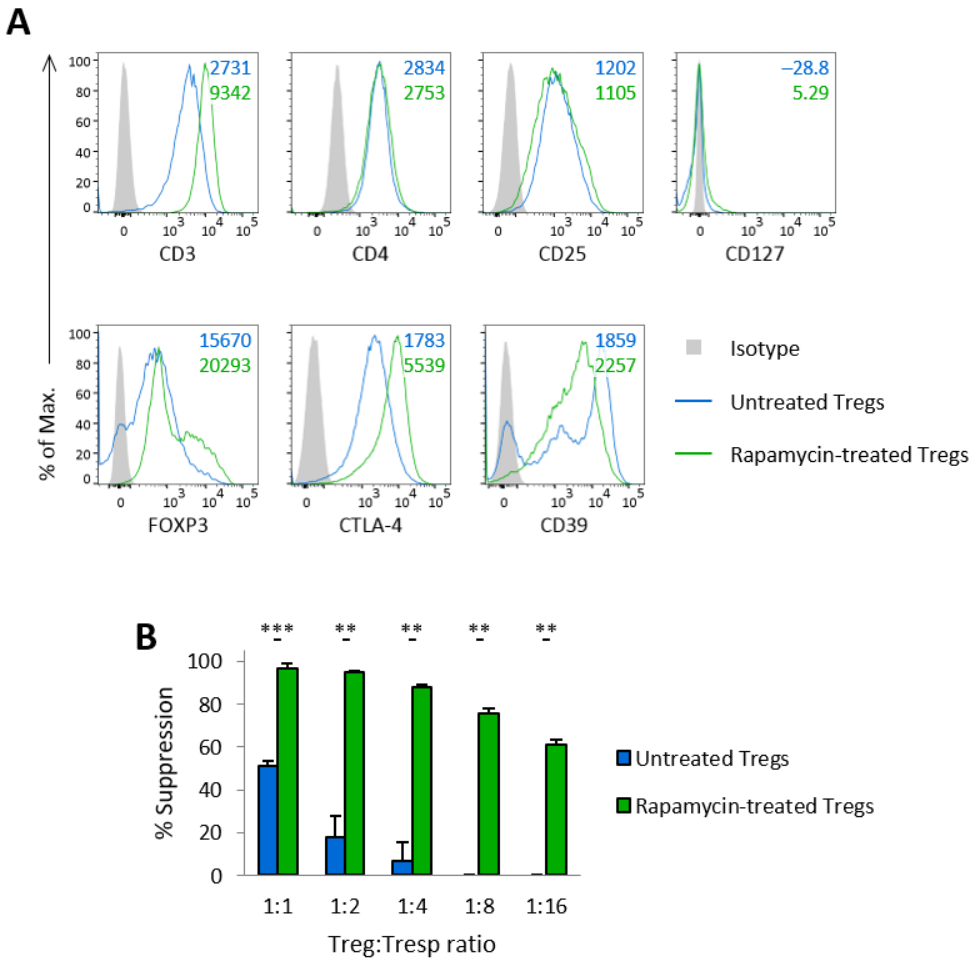

Figure 4-2 | **Human Tregs cultured in the presence of rapamycin were highly suppressive.** *A: Representative flow cytometry data comparing the expression of typical Tregs markers by untreated (blue line) and rapamycin-treated (green line) Tregs. Tregs were expanded in the presence or absence of 100 nM rapamycin for 20 days prior to analysis. Isotype control staining is shown in solid grey and MFI values are provided in the top right corner of each plot. Data representative of 4 individual experiments B: Suppressive capacity of untreated (blue bars) and rapamycin-treated (green bars) human Tregs. After 20 days of expansion in the presence or absence of rapamycin, Tregs were co-cultured with CTV-labelled Tresps which were activated in a polyclonal manner using a low-dose of anti-human CD3/CD28 beads (1:40 bead:Tresp ratio). Cells were co-cultured at varying ratios for 5 days after which Tresp proliferation was measured by CTV dilution using a flow cytometer. % suppression was calculated as the inverse of % Tresp proliferation, relative to Tresps cultured alone. Data shown is the mean + SD of 3 technical replicates and is representative of 3 individual experiments. Statistical significance was determined using a two-tailed paired Student's t-test where **=p<0.01 and ***=p<0.001.*

4.3.1.3 Human Tregs were lentivirally transduced to express the A2 CAR constructs with a high efficiency

As lentiviral delivery of the A2 CAR into human T cells was demonstrated to be more efficient than retroviral delivery (Chapter III), a similar approach was adopted to generate human A2 CAR Tregs. Human $CD4^+CD25^+$ Tregs were enriched from peripheral blood, activated using anti-human CD3/CD28 beads and cultured in the presence of 1,000 U/mL IL-2 and 100 nM rapamycin, as described above. To transduce Tregs in their optimum culture media, lentiviral particles were precipitated and re-suspended in X-vivo complete cell media (section 2.2.1). Furthermore, these viral particles were suspended in $^1/_{10}$ of their original volume to concentrate the viral particles 10-fold with the rationale that concentrated lentiviral particles may increase the chances of the CAR genes being successfully integrated into the genome of the Tregs, ultimately resulting in a better transduction efficiency.

The optimal viral particle concentration required to efficiently delivered the A2 CAR genes into human Tregs was determined by transducing these cells with varying concentrations of virus. Freshly-isolated Tregs were activated using anti-human CD3/CD28 beads and transduced after 72 hours with unconcentrated (1X), 2-fold concentrated (2X) or 4-fold concentrated (4X) viral particles, relative to the concentration of viral particles present in the original transfected HEK293T supernatant (Figure 4-3A). The efficiency with which the Tregs were transduced was measured by flow cytometry 7 days post-transduction whereby the proportion of $eGFP^+$ cells was assessed. Tregs which were incubated with 1X, 2X and 4X lentiviral particles were transduced with efficiencies of 52.7 ± 12.6%, 67.8 ± 11.2% and 79.5 ± 7.3% (n=4), respectively, demonstrating that the Tregs were transduced with higher efficiencies when higher concentrations of viral particles were used (Figure 4-3B). As such, a viral concentration of 4X was selected for downstream experiments.

The optimal time-point within the Treg expansion protocol (Figure 2-1) for transducing the Tregs was also investigated by transducing a fraction of the cells 72 hours after each stimulation. Freshly-isolated Tregs were activated using anti-human CD3/CD28 beads, cultured in the presence of rapamycin and re-stimulated with fresh anti-CD3/CD28 beads every 10 days. 72 hours after each stimulation, a sample of the activated Tregs was removed and transduced with 4X lentiviral particles, as detailed above. Transduced cells were maintained in culture for an additional 7 days before their transduction efficiency was assessed by flow cytometry (Figure 4-4A). The human Tregs were transduced with the highest efficiency following the first stimulation (79.7 ± 7.1%; n=4; Figure 4-4B), compared to the second (49.7 ± 6.8%) and third (59.2 ± 10.1%) stimulations.

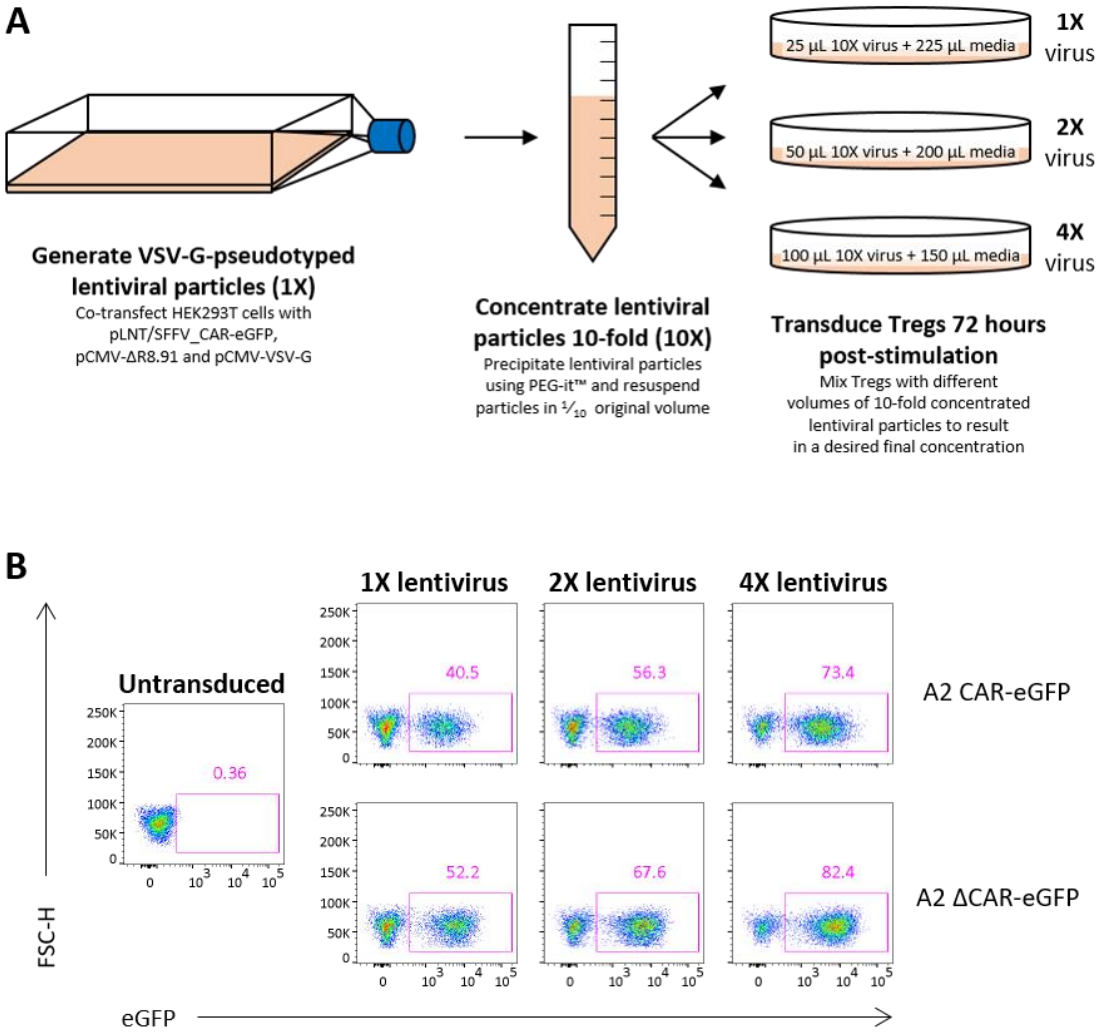

Figure 4-3 | **Transducing human Tregs with higher concentrations of VSV-G-pseudotyped lentiviral particles yielded a higher transduction efficiency.** *A: VSV-G-pseudotyped lentiviral particles were generated by co-transfecting HEK293T cells with pLNT/SFFV_CAR-eGFP, pCMV-Δ8.91 and pCMV-VSV-G plasmids. Transfected HEK293Ts were incubated for 48-56 hours, after which the supernatant containing viral particles was collected. The viral particles were precipitated and re-suspended in X-vivo complete cell media in one tenth of the volume that was harvested from the HEK293T cells. Consequently, the viral particles were concentrated 10-fold (10X). Human $CD4^+CD25^+$ Tregs were enriched from the peripheral blood of healthy donors, activated using anti-human CD3/CD28 beads and cultured in the presence of 1,000 U/mL IL-2 and 100 nM rapamycin. After 72 hours, $5x10^5$ Tregs were transduced with different concentrations of lentiviral particles in a 24-well plate. In each well, the cells were mixed with media and 10X virus such that a final viral concentration of 1X, 2X or 4X was achieved. These cells were maintained in culture for 7 days where fresh media, IL-2 and rapamycin was replenished every 2 days. B: The efficiency with which the Tregs were transduced was measured after 7 days by flow cytometry based on eGFP expression. Data representative of 2 individual experiments.*

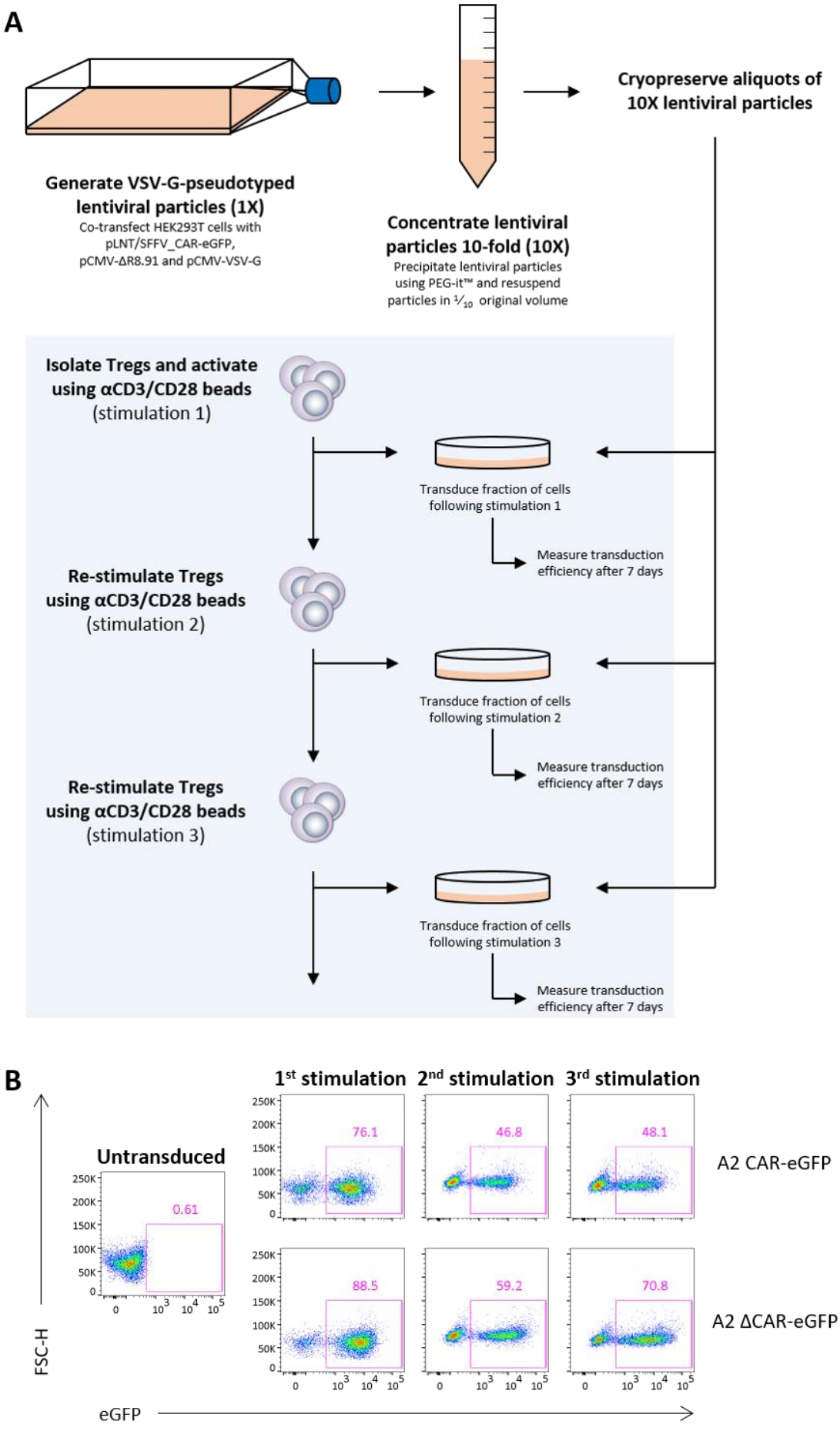

Figure 4-4 | **Human Tregs were transduced with VSV-G-pseudotyped lentiviral particles more efficiently at an earlier time-point.** *Human CD4⁺CD25⁺ Tregs were enriched from the peripheral blood of healthy donors,*

activated using anti-human CD3/CD28 beads and cultured in the presence of 1,000 U/mL IL-2 and 100 nM rapamycin. Tregs were re-stimulated with fresh anti-CD3/CD28 beads every 10 days. 72 hours after each stimulation, a fraction of the cells were transduced with 4X concentrated VSV-G-pseudotyped lentiviral particles to express either the A2 CAR-eGFP or A2 ΔCAR-eGFP construct. The efficiency of transduction was measured 7 days post-transduction by flow cytometry based on eGFP expression. Data representative of 2 individual experiments.

Overall, these results demonstrated that lentiviral transduction efficiently delivered the A2 CAR-eGFP and A2 ΔCAR-eGFP constructs into human Tregs. Furthermore, the optimal conditions for transducing the Tregs in this setting was after the first stimulation using a viral concentration of 4X. Both of these criteria were adopted for subsequent experiments.

4.3.2 Phenotypic and functional assessment of lentivirally transduced human Tregs

Lentiviral transduction is a safe gene delivery approach which is currently employed clinically to deliver CD19-specific CAR genes into target T cells for the treatment of CLL (Kalos, Levine et al. 2011, Porter, Levine et al. 2011). Lentiviruses have also been routinely used *in vitro* to genetically manipulate Tregs (Wright, Notley et al. 2009, Brusko, Koya et al. 2010, Plesa, Zheng et al. 2012, MacDonald, Hoeppli et al. 2016). However, given the nature of these viruses to integrate proviral into active regions of a target cells' genome (Ciuffi 2008), it was possible that transducing the Tregs using the protocol optimised above could have undesired effects on these cells. To assess this, untransduced and transduced Tregs were compared.

Human Tregs were isolated, activated in a polyclonal manner using anti-human CD3/CD28 beads and expanded in the presence of 1,000 U/mL IL-2 and 100 nM rapamycin. After 72 hours, these cells were transduced with 4X-concentrated VSV-G-pseudotyped lentiviral particles to express either the A2 CAR-eGFP or A2 ΔCAR-eGFP constructs and expanded for an additional 7 days. The average transduction efficiencies for the A2 CAR-eGFP and A2 ΔCAR-eGFP constructs was 55.1 ± 20.3% and 69.2 ± 16.4%, respectively (n=15). Interestingly, as well as being transduced more efficiently, the A2 ΔCAR-eGFP construct was also more highly expressed in the Tregs (MFI = 13,463 ± 8,572; n=19) compared to the A2 CAR-eGFP construct (MFI = 5,728 ± 3,772). This was likely due to the fact that the A2 ΔCAR-eGFP gene was 29% smaller than the A2 CAR-eGFP gene and smaller viral RNA species are more easily packaged and delivered into target cells (Cante-Barrett, Mendes et al. 2016).

Successfully transduced cells were isolated by cell sorting 7 days post-transduction, gating on eGFP$^+$ cells. eGFP expression was shown to be directly correlated with presence of the CAR on the cell surface, as detected by 9E10 cMyc tag epitope staining (Figure 4-5). The Tregs were then re-simulated with anti-human CD3/CD28 beads and expanded for an additional 10 days in the presence of 1,000 U/mL IL-2 and 100 nM rapamycin prior to characterisation.

4.3.2.1 The phenotype of human Tregs was unaltered following lentiviral transduction

At the end of the expansion protocol, more than 95% (95.3-97.8 ± 4.7%; n=4) of the transduced Tregs were eGFP$^+$, demonstrating that the A2 CAR-eGFP and A2 ΔCAR-eGFP genes were stably integrated and expressed in the Tregs. To investigate whether the expression of typical Treg markers was influenced by the transduction and cell sorting processes, the expression of CD4, CD25, CD127, FOXP3, CTLA-4 and CD39 in these cells was assessed (Figure 4-6). Untransduced, A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs all exhibited a similar phenotype; they remained

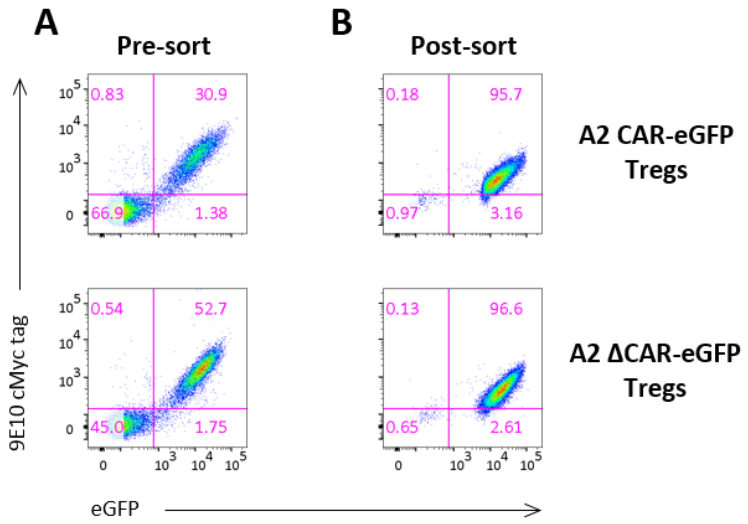

Figure 4-5 | **Human Tregs expressing the A2 CAR-eGFP and A2 ΔCAR-eGFP constructs were isolated with a high purity by cell sorting based on eGFP expression.** *Human Tregs cultured in the presence of rapamycin were lentivirally transduced with VSV-G-pseudotyped particles 3 days post-activation to express the A2 CAR-eGFP or A2 ΔCAR-eGFP constructs. Successfully transduced cells were isolated by cell sorting, based on their eGFP expression 7 days post-transduction. A: Transduction efficiency of human Tregs 7 days post-transduction, immediately before cell sorting. Cells were stained with a fluorescently-conjugated 9E10 clone antibody specific for the cMyc tag present in the extracellular domain of each A2 CAR construct prior to data acquisition. B: eGFP$^+$ cell purity immediately after cell sorting. Data shown is representative of 2 (A) or 15 (B) individual experiments.*

$CD4^+CD25^+CD127^{lo}$ with a high expression the transcription factor FOXP3 (94.2-95.8 ± 7.3%; n=4, Figure 4-6B). With regards to Treg functional markers, all of the cells expressed CTLA-4 (91.3-95.3 ± 3.1%) with a high proportion also expressing CD39 (72.4-81.7 ± 16.3%). Overall, no significant differences were observed in the expression of the Treg markers analysed between the 3 Treg cell lines.

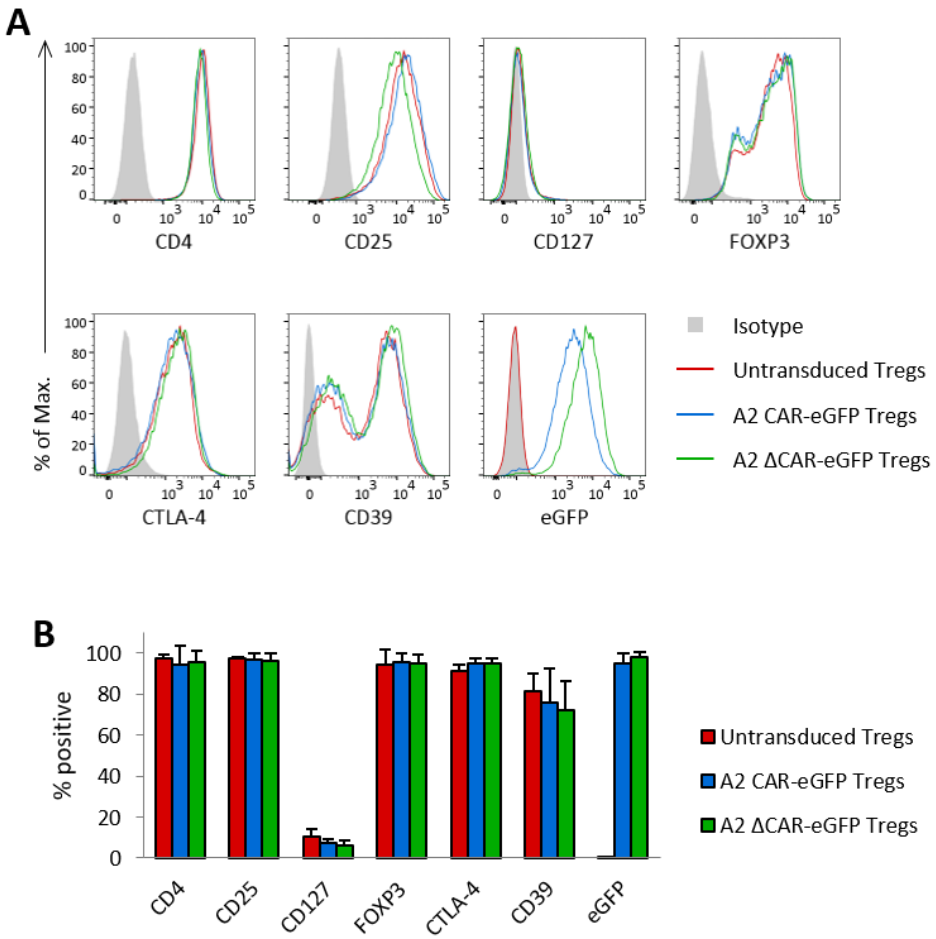

Figure 4-6 | **Human Tregs remain $CD4^+CD25^{hi}CD127^{lo}$ with a high expression of FOXP3, CTLA-4 and CD39 following lentiviral delivery of the A2 CAR-eGFP/ΔCAR-eGFP constructs.** *Cell sorted $eGFP^+$ Tregs were re-stimulated in a polyclonal manner using anti-human CD3/CD28 beads and cultured in the presence of rapamycin for 10 days. The expression of crucial human Treg markers was then measured by flow cytometry. Dead cells were excluded using a LIVE/DEAD dye and cells were stained with fluorescently-conjugated antibodies specific for CD4, CD25, CD127, FOXP3, CTLA-4 and CD39. Cells were fixed and permeabilised prior to staining of FOXP3 and CTLA-4 A: Representative flow cytometry data comparing the expression of the aforementioned Tregs markers and eGFP by untransduced (red line) A2 CAR-eGFP (blue line) and A2 ΔCAR-eGFP (green line) Tregs. Isotype control staining is shown in solid grey. B: Pooled flow cytometry data comparing the expression of Treg markers by untransduced (red bars), CAR (blue bars) and ΔCAR (green bars) Tregs. Data represents mean + SD pooled from 4 individual experiments. A paired Student's t-test was performed to determine statistical significance but no significance was observed.*

Considering the functional importance of Treg trafficking *in vivo*, the expression of specific homing receptors was also evaluated. No significant differences in the proportion of Tregs expressing these markers were observed between the untransduced, A2 CAR-eGFP and A2 ΔCAR-eGFP Treg lines (Figure 4-7). All of the Tregs expressed the skin homing molecule CCR4

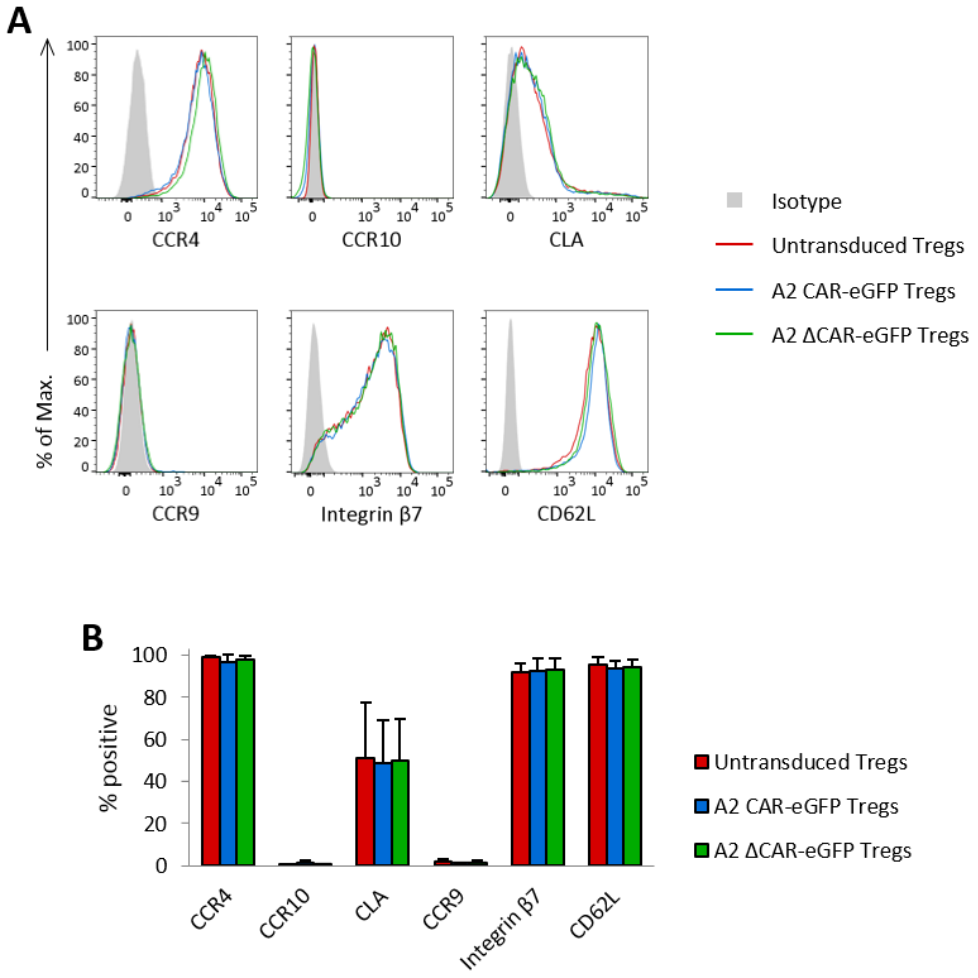

Figure 4-7 | **Human Tregs maintain their expression of specific homing receptors following lentiviral delivery of the A2 CAR-eGFP/ΔCAR-eGFP constructs.** *The expression of various homing receptors by the human Tregs was measured at the end of the expansion period by flow cytometry. Dead cells were excluded using a LIVE/DEAD dye and cells were stained with fluorescently-conjugated antibodies specific for the skin homing receptors CCR4, CCR10 and CLA, the gut-homing receptors CCR9 and Integrin β7, and the secondary lymphoid organ homing receptor CD62L. A: Representative flow cytometry data comparing the expression of the aforementioned homing receptors by untransduced (red line) A2 CAR-eGFP (blue line) and A2 ΔCAR-eGFP (green line) Tregs. Isotype control staining is shown in solid grey. B: Pooled flow cytometry data comparing the expression of homing receptors by untransduced (red bars), A2 CAR-eGFP (blue bars) and A2 ΔCAR-eGFP (green bars) Tregs. Data shown is mean + SD pooled from 4 individual experiments. A paired Student's t-test was performed to determine statistical significance but no significance was observed.*

(96.7-98.8 ±3.2%; n=4, Figure 4-7B) whilst an intermediate proportion expressed CLA (48.4-51.0 ± 26.4%) and few cells expressed CCR10 (0.6-1.5 ± 0.7%). Furthermore, a high proportion of the Tregs expressed the secondary lymphoid organ homing molecule CD62L (93.7-95.6 ± 3.9%). With regards to gut homing, whilst a high proportion of cells expressed integrin β7 (91.7-93.2 ± 5.9%), few cells expressed CCR9 (1.1-1.7 ± 1.0%) which is required for trafficking to gut-associated lymphoid tissues (Svensson, Marsal et al. 2002).

Overall, these results demonstrated that the phenotype of the rapamycin-treated Tregs remained stable following the transduction process, with a dominance of chemokine receptors favouring homing to the skin and lymph nodes, as previously published (Scotta, Esposito et al. 2013).

4.3.2.2 Human Tregs remained suppressive following lentiviral transduction

To investigate whether the lentiviral transduction procedure influenced the suppressive capacity of rapamycin-treated Tregs, the suppressive capacity of the A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs was compared to untransduced Tregs, as described above. Tresp proliferation was

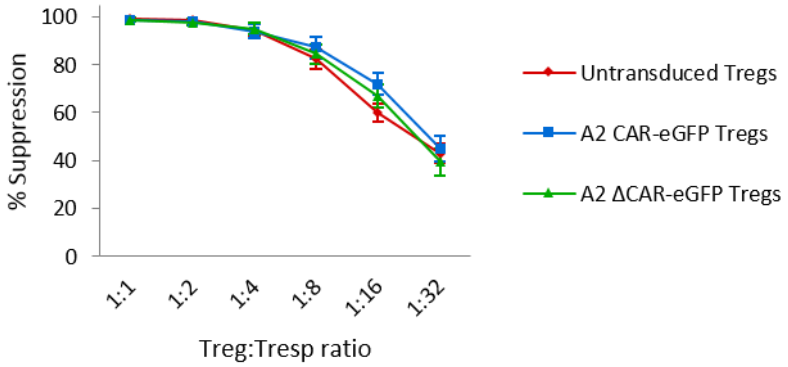

Figure 4-8 | **Human Tregs maintained their suppressive capacity following lentiviral delivery of the A2 CAR-eGFP/ΔCAR-eGFP constructs.** *The suppressive capacity of the human Tregs was assessed at the end of the Treg expansion period. Untransduced (red line), A2 CAR-eGFP (blue line) and A2 ΔCAR-eGFP (green line) Tregs were co-cultured with 1x10^5 autologous CTV-labelled CD4$^+$CD25$^-$ Tresps which were activated with a low-dose (1:40 bead:cell ratio) of anti-human CD3/CD28 beads. Tresp proliferation was measured after 5 days of culture. Dead cells were excluded using a LIVE/DEAD dye and CTV dilution was assessed using a flow cytometer. Tresp proliferation was calculated as a percentage relative to Tresps cultured in the absence of Tregs and the inverse of this % proliferation was used to determine the % suppression. Data shown represents mean ± SEM pooled from 5-6 individual experiments. A two-tailed paired Student's t-test was performed to determine statistical significance but no significant differences were observed.*

measured by CTV dilution and calculated using division indices. At all Treg:Tresp ratios analysed, no significant differences were observed in the extent to which the untransduced, A2 CAR-eGFP or A2 ΔCAR-eGFP suppressed Tresp proliferation (Figure 4-8). Furthermore, the overall suppressive profile of all 3 Treg lines was very similar. At a 1:32 ratio, untransduced Tregs inhibited Tresp proliferation by 43.0% ± 4.1% whilst the A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs suppressed the proliferation of the Tresps by 44.8% ± 5.4% and 39.4% ± 5.7%, respectively (n=5). Overall, these results demonstrated that delivery of the A2 CAR genes into human rapamycin-treated Tregs using second-generation VSV-G-pseudotyped lentiviral particles did not impede the suppressive ability of these cells.

4.4 DISCUSSION

In this chapter, a previously established GMP-compatible protocol was employed to enrich human $CD4^+CD25^+$ Tregs with an efficiency of approximately 90%. The sequential negative and positive selection protocol used was directly relatable to what is currently employed in clinical trials based at Guy's Hospital. These cells were activated in a polyclonal manner using anti-human CD3/CD28 beads and expanded in the presence of 1,000 U/mL IL-2, with or without 100 nM rapamycin. Rapamycin-treated Tregs were shown to express higher levels of FOXP3, CTLA-4 and CD39 and suppress autologous $CD4^+CD25^-$ Tresp proliferation more effectively than untreated Tregs, in line with previous studies (Putnam, Brusko et al. 2009, Golovina, Mikheeva et al. 2011, Scotta, Esposito et al. 2013).

Culturing Tregs in the presence of rapamycin also enhances the stability of these cells. Over the past few years, there has been a shift in the understanding of $CD4^+$ T cell subsets, moving away from the idea of defined lineages and towards a model based on T cell plasticity. This train of thought has emerged from studies which have demonstrated that $CD4^+$ T cells which exhibit typical characteristics of one subset (including specific transcription factor expression) can convert and exhibit characteristics of another subset. This is exemplified by the crosstalk which exists between tTregs and Th17 cells; culturing Th17 cells in the presence of TGFβ promotes conversion towards a regulatory phenotype (Bettelli, Carrier et al. 2006, Zhou, Lopes et al. 2008, Gagliani, Amezcua Vesely et al. 2015) whilst culturing Tregs in the presence of IL-6, IL-1β and IL-23 can cause a loss of FOXP3 expression and increased IL-17 production (Koenen, Smeets et al. 2008, Yang, Nurieva et al. 2008). However, studies have also demonstrated that culturing Tregs in the presence of rapamycin confers a resistance to this conversion (Golovina, Mikheeva et al. 2011, Tresoldi, Dell'Albani et al. 2011, Scotta, Esposito et al. 2013). This resistance

is believed to be due to changes in the epigenetic profile of the Tregs which promotes the expression of FOXP3 (Abele-Ohl, Leis et al. 2010, Golovina, Mikheeva et al. 2011, Rossetti, Spreafico et al. 2015). Additional studies have also demonstrated a link between this increased Treg stability and TSDR demethylation (Golovina, Mikheeva et al. 2011, Rossetti, Spreafico et al. 2015).

The results presented in this chapter proceeded to demonstrate that lentiviral transduction of the human Tregs did not influence the phenotype or suppressive ability of these cells. This is in line with previous studies which have shown similar findings (Hombach, Kofler et al. 2009, Brusko, Koya et al. 2010, Plesa, Zheng et al. 2012, Kim, Zhang et al. 2015, MacDonald, Hoeppli et al. 2016) but was important to address given the different laboratory conditions under which this gene manipulation was being performed. Previously published Treg transduction protocols transduce the cells 48-72 hours following polyclonal activation (Hombach, Kofler et al. 2009, Brusko, Koya et al. 2010). In this chapter, Tregs were activated in a polyclonal manner and cultured in the absence of IL-2 for 48 hours, allowing time for the rapamycin to act on the Tconvs. This concept is currently applied for an extended period of time (>4 days) in the GMP facility at Guy's Hospital. The Treg cultures were then supplemented with 1,000 U/mL IL-2 for 24 hours before being transduced with concentrated lentiviral particles 72 hours post-activation, in line with previous studies (MacDonald, Hoeppli et al. 2016). Transduction did not influence the expression of published Treg markers such as CD4, CD25, CD127, FOXP3, CTLA-4 and CD39, nor the expression of various homing receptors including CCR4, CCR10, CLA, CCR9, integrin β7 and CD62L. Furthermore, the suppressive capacity of the transduced Tregs was the same as the untransduced Tregs.

The adaptation of gene-modified Tregs in the clinic is somewhat dependent on current clinical trials, designed to assess the safety and efficacy of polyclonal Treg therapy. In these trials, promising results have been noted to date where Treg doses of up to $10x10^6$ cells/kg have been well tolerated in renal transplant recipients at Guy's Hospital. However, to apply CAR technology in this setting, it will be necessary to genetically modify the Tregs, a process which is associated with additional risks. Due diligence would dictate that a sequential timeline is employed whereby Treg therapy using unmodified cells is deemed safe before trials using gene-modified cells are initiated. This may unfortunately hamper the progression of clinical trials which aim to investigate the safety and efficacy of CAR Treg therapy.

4.4.1 SUMMARY

The results presented in this chapter confirmed that culturing human Tregs in the presence of rapamycin enhanced the functionality of these cells. Furthermore, these Tregs can be transduced lentivirally to express the A2 CAR constructs without influencing their phenotype or suppressive capacity.

CHAPTER V

CHACTERISATION OF HUMAN REGULATORY T CELLS EXPRESSING A HLA-A2-SPECIFIC CHIMERIC ANTIGEN RECEPTOR

5.1 INTRODUCTION

5.1.1 CHARACTERISING THE FUNCTIONAL ADVANTAGE OF CAR EXPRESSION IN HUMAN TREGS

Conferring antigen-specificity onto Tregs can influence the functionality of these cells in a variety of different ways. The most profound and archetypical effect is the efficacy with which these cells elicit a suppressive function in the presence of their target antigen. This is typically assessed using an "antigen-specific suppression assay" whereby $CD4^+CD25^-$ Tresps are activated with allogeneic APCs and the ability of the Tregs to inhibit Tresp proliferation is assessed (Thornton and Shevach 2000, Tang, Henriksen et al. 2004, Zhou, Borojevic et al. 2004, Fujio, Okamoto et al. 2006, Tsang, Tanriver et al. 2008, Stephens, Malpass et al. 2009, Tsang, Tanriver et al. 2009, Wright, Notley et al. 2009, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). Variations on this type of experiment are included in all studies investigating the influence of antigen-specificity on Treg function.

Trafficking of Tregs is believed to be principally driven by homing receptors, although studies investigating antigen-specific Tconvs have demonstrated that this process can be influenced by interactions made through the TCR. This is primarily mediated through the TCR engaging MHC-peptide complexes which are presented by epithelial cells. In a transplant context, this engagement typically leads to the induction of anergy (Marelli-Berg, Weetman et al. 1997). However, studies have demonstrated that TCR engagement can also facilitate the egression of T cells (Marelli-Berg, Frasca et al. 1999, Greening, Tree et al. 2003, Marelli-Berg, James et al. 2004) and Tregs (Fu, Kishore et al. 2014) into specific tissues. TCR engagement may also contribute to the retention of antigen-specific T cells in tissues where a cognate antigen is expressed, as has been observed in models of MS (Karin, Szafer et al. 1993) and T1D (Lennon, Bettini et al. 2009).

5.1.2 HUMANISED MOUSE MODELS IN TRANSPLANTATION

Mouse models have been instrumental in progressing our understanding of the immune system. However, treatment options which have been revealed in mice have not always been successfully translated to humans due to fundamental differences which exist between these two organisms. A prime example relates to non-obese diabetes (NOD) mice which spontaneously develop T1D (Makino, Kunimoto et al. 1980). Studies over the past 35 years have attributed this to mutations in various genetic loci including the MHC loci (Lund, O'Reilly et al. 1990, Miyazaki, Uno et al. 1990, Slattery, Kjer-Nielsen et al. 1990, Hattori, Yamato et al. 1999), the *preproinsulin* gene (French, Allison et al. 1997, Todd and Wicker 2001) and a locus comprising the *CD28* and *CTLA-4* genes

(Hill, Lyons et al. 2000, Lamhamedi-Cherradi, Boulard et al. 2001). Studies performed with these mice demonstrated that treatment with rapamycin and IL-2 prevented the spontaneous development and recurrence or diabetes after islet transplantation (Rabinovitch, Suarez-Pinzon et al. 2002). However, when a similar approach was adopted in a clinical trial, rapamycin and IL-2 treatment was found to have the opposite effect in humans, exacerbating β-cell dysfunction and overall having a detrimental effect (Long, Rieck et al. 2012).

The issues surrounding the discordance between different species can be partially circumvented through the use of humanised mouse models whereby mice are engrafted with human immune cells. In order to achieve engraftment, it is necessary to use immunocompromised mice such that transferred human cells are not immediately killed by xeno-immune responses. This was a particular issue in early humanised mouse models which relied on mutations in the protein kinase, DNA activated, catalytic polypeptide, *scid* (*Prkdc*^{scid}) gene, where injection of haematopoietic cells or PBMCs commonly resulted in <0.1% engraftment (McCune, Namikawa et al. 1988, Mosier, Gulizia et al. 1988, Lapidot, Pflumio et al. 1992). However, this was resolved over the forthcoming years with the development of mice with alternative genetic mutations (Goldman, Blundell et al. 1998, Mazurier, Fontanellas et al. 1999, Shultz, Lyons et al. 2005). Consequently, humanised mice are now utilised to investigate human immune responses *in vivo* in a wide variety of settings, ranging from solid and haematological cancers to autoimmunity and transplantation.

Two strains which are commonly used in modern humanised mouse models are BRG and NSG mice. BRG mice lack the RAG2 and the γ_c gene (section 3.1.2) and as such, do not develop cells which rely on rearrangement of the TCR/BCR loci or on detection of IL-2, IL-4, IL-7, IL-9, IL-15 or IL-21 (Sugamura, Asao et al. 1996, Shultz, Lyons et al. 2005). Along similar lines, NSG mice combine the features of NOD and *scid* mice with the deficiencies which accompany removal of the γ_c gene. The mutations in both BRG and NSG mice culminate in strains which lack lymphoid lineage-derived cells, namely B cells, T cells and NK cells (Cao, Shores et al. 1995, DiSanto, Muller et al. 1995, Ohbo, Suda et al. 1996).

Unlike many of their predecessors, BRG and NSG mice support long-term reconstitution of human immune cells, making them versatile tools which can be used in many research settings (Shultz, Lyons et al. 2005). However, the range and location of the human cells which are reconstituted is dependent on many factors including the nature of the cells transferred, the route of administration and the age of the mice. For example, mice which receive human haematopoietic stem cells (HSC) are capable of reconstituting a more complete immune system than mice which receive PBMCs (Shultz, Lyons et al. 2005, Brehm, Cuthbert et al. 2010, Ali, Flutter et al. 2012).

Furthermore, HSC infusion into new-born mice achieves a more efficient engraftment than infusion into adult mice (Brehm, Cuthbert et al. 2010).

Despite advances with humanised mouse models, there are still certain issues which remain to be addressed. For example, the T cells which develop in the majority of these models do not undergo thymic selection which has important implications when assessing the functionality of T cells or Tregs. Recent studies have attempted to address this using different approaches. One approach is to reconstitute irradiated immunocompromised mice with human HSCs and allow the T cells to develop within the mouse thymic environment (Lepus, Gibson et al. 2009, Brehm, Cuthbert et al. 2010). Although this can be accomplished with relative ease, it is important to note that the nature with which human T cells engage murine MHC-peptide complexes is not fully understood (Halkias, Yen et al. 2015). However, this approach was recently improved by Serra-Hassoun *et al*. who generated mice in which murine MHC presentation was replaced with human HLAs, allowing human HSCs to be educated against human HLA complexes (Serra-Hassoun, Bourgine et al. 2014). An alternative approach to generate humanised mice with thymically educated human T cells is to surgically implant adult mice with a human foetal thymus and liver under the kidney capsule and then to transfer human HSCs into these mice (Lan, Tonomura et al. 2006, Aryee, Shultz et al. 2014). This is known as the BM, liver and thymus (BLT) model and achieves both high levels of human cell engraftment and yields T cells which have been educated in a human thymus (Safinia, Becker et al. 2016).

In the transplant field, humanised mouse models have been used to investigate both GvHD and solid tissue transplantation. An overview of these applications is provided below.

5.1.2.1 Xeno-graft-versus-host disease

BM transplantation (or more broadly haematopoietic stem cell transplantation (HSCT)) is a treatment option which is performed to treat various haematological conditions including cancers (leukaemias/myelomas), aplastic anaemia and congenital immunodeficiencies (Safinia, Becker et al. 2016). However, the benefits of this treatment option are severely compromised by GvHD in which the transferred donor immune cells recognise their recipient surroundings as foreign.

To research GvHD *in vivo*, humanised mouse models have been developed which rely on a xeno-immune response elicited by human immune cells towards murine antigens. In these models, human PBMCs are usually transferred into adult recipient mice (Ali, Flutter et al. 2012). Over the past 20 years, it has become apparent that higher levels of engraftment are achievable

in mice which are more severely immunocompromised, such as NSGs (Ito, Katano et al. 2009) or mice which have undergone TBI to kill haematopoietic progenitor cells (Cui, Hisha et al. 2002, van Rijn, Simonetti et al. 2003). A recent study performed in collaboration with the host laboratory demonstrated that human cell engraftment and GvHD development was faster in NSG mice than BRG mice (Ali, Flutter et al. 2012). Studies have also demonstrated that the GvHD symptoms induced by the transfer of human PBMCs can be suppressed by the co-transfer of Tregs. This suppression is mediated in a dose-dependent manner and has been exploited to both demonstrate the efficacy of Treg therapy, and the potential of biological agents which promote the activity of Tregs *in vivo* (Mutis, van Rijn et al. 2006, Becker, Taube et al. 2009, Hahn, Stahl et al. 2013, Hahn, Bellinghausen et al. 2015).

5.1.2.2 Solid tissue transplantation

Various healthy human tissues can be transplanted into mice such as vessels (Lorber, Wilson et al. 1999, Koh, Wang et al. 2004, Nadig, Wieckiewicz et al. 2010), islets (Jacobson, Heuts et al. 2010, Wu, Hester et al. 2013, Xiao, Ma et al. 2014) and bronchi (Sommer, Knofel et al. 2015). However, the most common human tissue transplanted onto mice is human skin due to the relative abundance of this organ and the less invasive nature of the transplant procedure (Issa, Hester et al. 2010, Racki, Covassin et al. 2010, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013).

Early human skin xenograft transplant models were performed using C.B-17 *scid* recipient mice and were deemed highly successful as the skin xenografts maintained their tissue architecture (Kawamura, Niguma et al. 1992, Alegre, Peterson et al. 1994, Murray, Petzelbauer et al. 1994). However, human immune cells did not efficiently engraft in these mice. To address this, a range of different strategies were trialled with varying success; some models opted to increase the number of cells which were injected into the recipient mice (up to $3x10^8$/mouse) whilst other models investigated using TBI or different recipient mouse strains (Alegre, Peterson et al. 1994, Murray, Petzelbauer et al. 1994).

Current models opt to use NSG or BRG mice as human skin xenograft recipients and to mediate graft rejection through the transfer of human allogeneic PBMCs (Issa, Hester et al. 2010, Racki, Covassin et al. 2010, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). However, the functional granulocytes in these mice have been shown to significantly damage the tissue architecture thus consistent depletion of these cells achieved through bi-weekly injections of anti-mouse Gr-1 is necessary (Racki, Covassin et al. 2010). Transfer of human PBMCs into Gr-1-depleted mice bearing well-healed human skin xenografts resulted in alloimmune-mediated graft injury which can be

measured by a loss of microvasculature and destruction of the epidermal/dermal skin layers (Racki, Covassin et al. 2010, Sagoo, Ali et al. 2011). This damage correlateed with human immune cell infiltration and was alleviated by the co-transfer of human Tregs (Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). Importantly, the latter studies also demonstrated that allograft-specific Tregs, generated by expanding Tregs using allogeneic APCs, offered significantly more protection from alloimmune-mediated graft injury than polyclonal Tregs, upon adoptive transfer. In an islet transplant setting, Treg transfer was demonstrated to delay allograft rejection and diabetes onset by inhibiting innate immune cell infiltration and T cell differentiation/proliferation (Wu, Hester et al. 2013, Xiao, Ma et al. 2014).

5.2 AIMS AND OBJECTIVES

The aim of this chapter was to assess how expression of an alloantigen-specific CAR influenced the functionality of human Tregs *in vitro* and *in vivo*. The functionality of the Tregs was confirmed *in vitro* through the use of an antigen-specific suppression assay with allogeneic HLA-A2^{+} or HLA-A2^{-} B-LCLs as APCs. The trafficking of these cells was then assessed *in vitro* using a transmigration system whereby the Tregs were to be flowed across HLA-A2^{+} or HLA-A2^{-} endothelial HUVEC monolayers. Having confirmed that the A2 CAR-eGFP Tregs were functionally superior *in vitro*, the protective efficacy of these cells was compared to polyclonal and A2 ΔCAR-eGFP Tregs *in vivo* in both a xeno-GvHD model using HLA-A2 transgenic mice and a human skin xenograft transplant model.

5.3 RESULTS

5.3.1 HUMAN TREGS EXPRESSING THE A2 CAR-EGFP OR A2 ΔCAR-EGFP SPECIFICALLY RECOGNISED HLA-A2

In the analyses performed in Chapter IV, it was confirmed that lentiviral delivery of the A2 CAR genes into rapamycin-treated Tregs did not influence the phenotype or suppressive capacity of these cells following polyclonal stimulation. However, in order to confirm whether CAR expression conferred an ability to recognise HLA-A2, transduced Tregs were stained with dextramers which presented peptides in the context of HLA-A2 or HLA-B7 (an irrelevant control). These dextramers were kindly donated by Dr Marc Martinez-Llordella (Institute of Liver Sciences,

King's College London, UK). Given the TCR heterogeneity of the polyclonally-expanded cells, no polyclonal (untransduced) Tregs were able to recognise the specific HLA-peptide complexes presented in these dextramers (Figure 5-1A). In contrast, all eGFP$^+$ Tregs expressing the A2 CAR-eGFP (Figure 5-1B) and A2 ΔCAR-eGFP (Figure 5-1C) constructs were able to recognise the HLA-A2-based dextramer but not the irrelevant HLA-B7-based dextramer. Furthermore, as alluded to above (Figure 4-5), Tregs which expressed more of the CAR constructs (a higher MFI) were able to bind a greater amount of the HLA-A2-based dextramer, demonstrating a direct correlation between CAR expression and HLA-A2 recognition. Overall, these results confirmed that Tregs expressing the A2 CARs specifically recognised intact HLA-A2-peptide complexes.

To demonstrate the activation of A2 CAR-eGFP Tregs in the presence of HLA-A2, transduced Tregs were co-cultured with artificial HLA-A2$^+$ or HLA-A2$^-$ APCs for 18 hours after which Treg expression of CD69 expression was measured by flow cytometry. Two forms of APC were used: K562 cells and EBV-transformed B-Lymphoblastoid cell lines (B-LCL). K562 cells are immortalised myelogenous leukaemia cells which do not express MHC molecules (Andersson, Nilsson et al. 1979). The K562 cell lines used were kindly donated by Dr Marc Martinez-Llordella and were either empty (HLA-A2$^-$) or stably transduced to express HLA-A2 (Figure 5-2A). B-LCLs are B cells which are derived from HLA-typed donors and immortalised by CMV transduction (Leung, Maurer

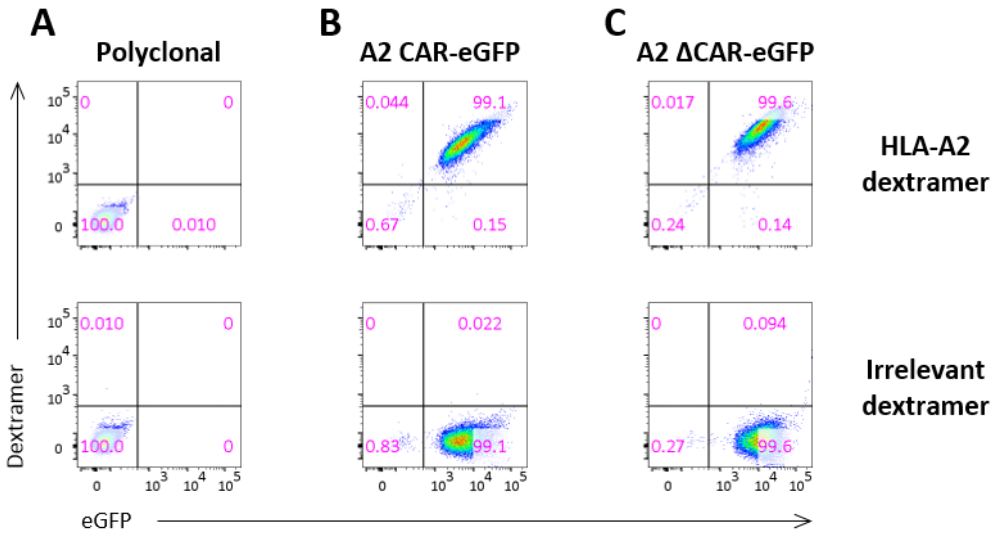

Figure 5-1 | **Lentiviral delivery of the A2 CAR-eGFP/ΔCAR-eGFP constructs into human Tregs confers the ability to specifically recognise HLA-A2.** *The ability of the human Tregs to specifically recognise HLA-A2 was determined by dextramer staining at the end of the Treg expansion period. Polyclonal (A), A2 CAR-eGFP (B) and A2 ΔCAR-eGFP (C) Tregs were stained with PE-conjugated dextramers which presented peptides in the context of HLA-A2 or HLA-B7 (irrelevant dextramer) and dextramer recognition was measured by flow cytometry.*

et al. 2013). The B-LCLs used were SPO (HLA-A2$^+$) or BM21 (HLA-A2$^-$) which both expressed the MHC class II molecules HLA-DR11 (Figure 5-2B). Tregs were co-cultured with irradiated (120 Gy) APCs at a 4:1 Treg:APC ratio for 18 hours. Total cells were then harvested, stained with fluorophore-conjugated antibodies specific for CD4 and CD69 and expression of CD69 was measured by flow cytometry. As shown in Figure 5-2, polyclonal and A2 ΔCAR-eGFP Tregs were not activated upon co-culture with either the HLA-A2$^+$ or HLA-A2$^-$ APCs. In contrast, CD69 was upregulated on the A2 CAR-eGFP Tregs following co-culture with the HLA-A2$^+$ APCs only.

A similar experimental approach was adopted to assess whether proliferation was induced in A2 CAR-eGFP Tregs upon co-culture with HLA-A2$^+$ APCs. Tregs were co-cultured at a 1:1 ratio with the irradiated artificial APCs described above for 72 hours. 1 µCi ^{3}H-thymidine was added for the last 18 hours of culture and proliferation was measured as ^{3}H-thymidine incorporation using a beta-plate liquid scintillation counting approach. A2 CAR-eGFP Tregs proliferated significantly more in the presence of HLA-A2-transfected K562s than in the presence of HLA-A1-transfected K562s (p=0.029; Figure 5-3A, representative of 2 experiments). This difference was not observed for the polyclonal (p=0.15) or A2 ΔCAR-eGFP (p=0.58) Tregs. Similar results were obtained when

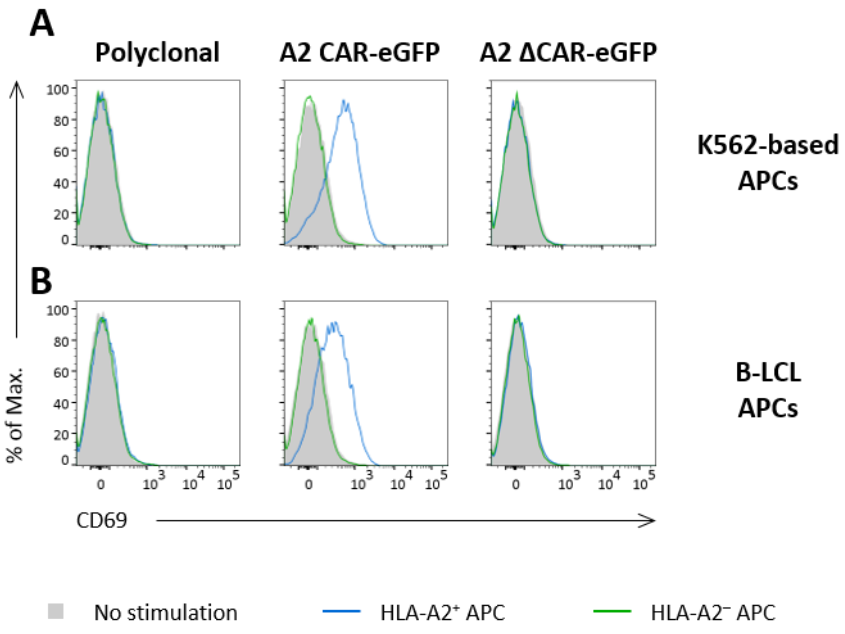

Figure 5-2 | **A2 CAR-eGFP Tregs were specifically activated in the presence of HLA-A2.** *Tregs were co-cultured at the end of their expansion period with irradiated (120 Gy) artificial APCs for 18 hours at a 4:1 Treg:APC ratio after which CD69 expression in the CD4$^+$ Tregs was analysed by flow cytometry. The APCs used were K562s (A) or B-LCLs (B). The K562s were either empty (HLA-A2$^-$; green line) or stably transfected to express HLA-A2 (blue line). The B-LCLs used were SPO (HLA-A2$^+$; blue line) or BM21 (HLA-A2$^-$; green line). Tregs cultured without APCs are shown in solid grey. Data representative of 2 individual experiments.*

the Tregs were co-cultured with HLA-A2$^+$ (SPO) and HLA-A2$^-$ (BM21) B-LCLs whereby A2 CAR-eGFP Tregs proliferated more in the presence of HLA-A2 (p=0.0047; Figure 5-3B, representative of 2 experiments) whilst polyclonal (p=0.74) and A2 ΔCAR-eGFP (p=0.76) Tregs did not.

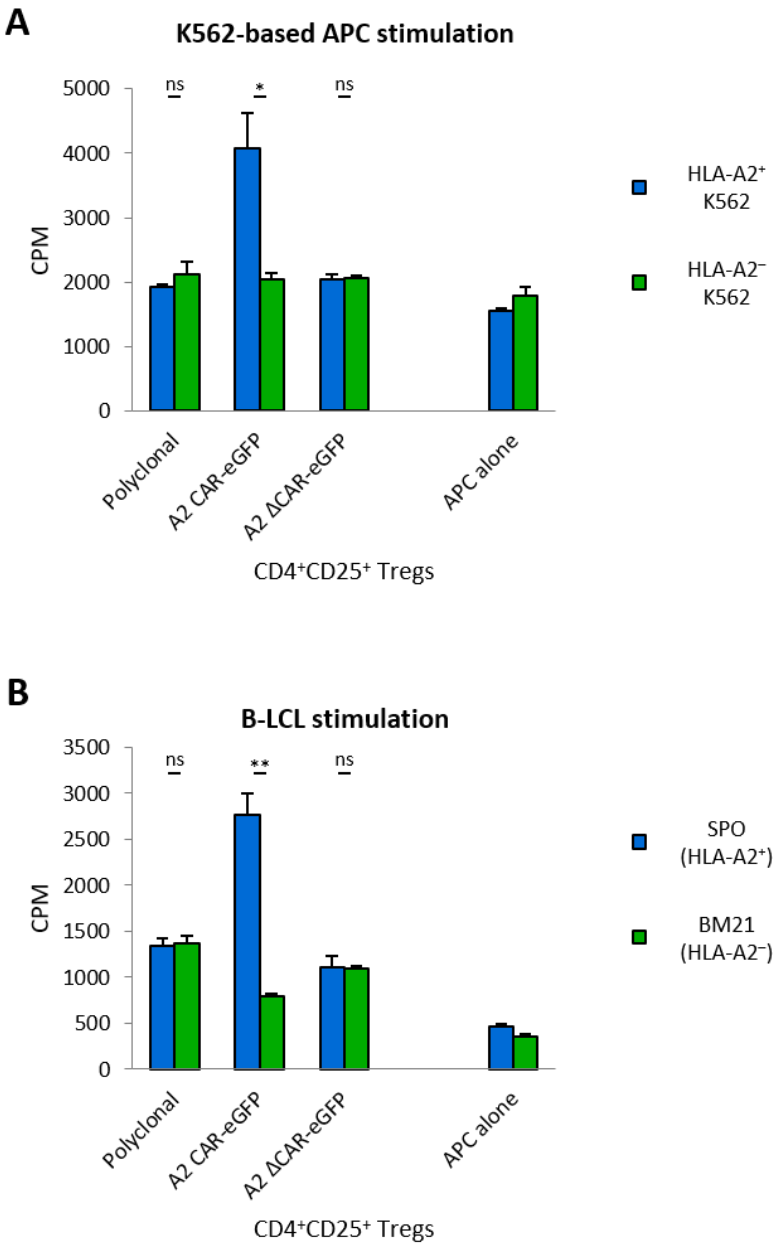

Figure 5-3 | **A2 CAR-eGFP Tregs were induced to proliferate in the presence of HLA-A2.** *Tregs were co-cultured at the end of their expansion period with irradiated (120 Gy) artificial APCs for 18 hours at a 1:1 Treg:APC ratio after which Treg proliferation was measured by ^{3}H-thymidine incorporation. The APCs used were either K562s (A) or B-LCLs (B). The K562s were stably transfected to express either HLA-A2 (blue bars) or HLA-A1 (green bars). The B-LCLs used were SPO (HLA-A2$^+$; blue bars) or BM21 (HLA-A2$^-$; green bars). Data shown is mean + SD of technical triplicates and is representative of 2 individual experiments. Statistical significance was determined using a two-tailed paired Student's t-test where *p<0.05 and **p<0.01. ns = not significant.*

Overall, these results demonstrated that Tregs expressing either of the A2 CARs were able to specifically recognise HLA-A2. However, Treg activation following HLA-A2 engagement was dependent on the ability of the CAR construct to signal intracellularly as no activation was observed for Tregs expressing the A2 ΔCAR-eGFP construct which lacked an intracellular CD28-CD3ζ signalling domain.

5.3.2 Assessment of the efficacy of human A2 CAR-eGFP Tregs *in vitro*

Having confirmed that human Tregs expressing the A2 CARs were specifically activated in the presence of HLA-A2, the next step was to assess whether this conferred a conferred a functional advantage to these cells in the presence of HLA-A2. This was accomplished through the use of various Treg functional assays which were performed in the presence and absence of HLA-A2.

5.3.2.1 Human A2 CAR-eGFP Tregs exhibited a superior suppressive capacity in the presence of HLA-A2

In a typical direct alloimmune response, $CD4^+$ T cells are activated by allogeneic APCs which present intact donor HLA-peptide complexes on their surface. To investigate whether A2 CAR-eGFP Tregs were more potent than polyclonal Tregs at suppressing Tresp activation and proliferation in the presence of HLA-$A2^+$ APCs, an antigen-specific suppression assay was performed using the aforementioned B-LCLs as APCs (Figure 5-4). CTV-labelled $CD4^+CD25^-$ Tresps were co-cultured with either HLA-$A2^+$ B-LCLs (DBB or SPO) or HLA-$A2^-$ B-LCLs (MOU or BM21) where the HLA-DR molecule expressed by the B-LCLs was the same (HLA-DR7 or HLA-DR11). Activation of the Tresp was facilitated by direct allorecognition of the HLA-DR molecules presented by the APCs and polyclonal, A2 CAR-eGFP or A2 ΔCAR-eGFP Tregs were added to these co-cultures to investigate the efficacy with which they could suppress this Tresp activation. As the Tregs were autologous to the Tresps, it was expected Tregs which had direct allospecificity would be activated through their TCR, in a similar manner to the Tresps (Veerapathran, Pidala et al. 2011). Consequently, polyclonal Tregs were expected to have the capacity to suppress Tresp activation but the efficacy with which this could be achieved was hypothesised to be greater for A2 CAR-eGFP Tregs in the presence of HLA-A2.

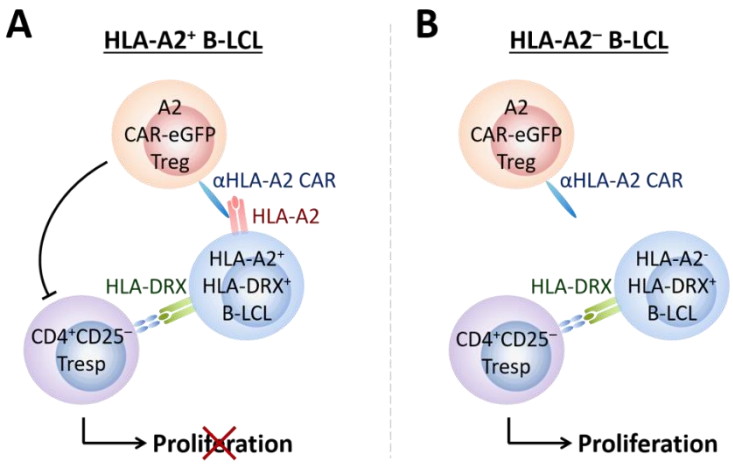

Figure 5-4 | **Schematic diagram explaining the setup of the antigen-specific suppression assay.** *Antigen-specific suppression assays were performed by co-culturing CD4$^+$CD25$^-$ Tresps (purple) with allogeneic B-LCLs as APCs (blue) and Tregs (orange). Tresp activation relied on direct allorecognition of the allogeneic HLA-DR molecule which was either HLA-DR7 or HLA-DR11 (green) and proliferation of these cells was measured by CTV dilution. Assays were performed in pairs which differed by the B-LCL which was included in the co-culture. In each pair, the B-LCLs expressed the same HLA-DR molecule but one B-LCL was HLA-A2$^+$ (A) and the other was HLA-A2$^-$ (B). Control Tresps were cultured with APCs only without Tregs. Polyclonal, A2 CAR-eGFP or A2 ΔCAR-eGFP Tregs were added to the Tresp:APC co-culture at Treg:Tresp ratios ranging from 1:2 to 1:128. It was hypothesised that A2 CAR-eGFP Tregs would supress the Tresp proliferation more effectively in the presence of HLA-A2 (A) compared to the absence of HLA-A2 (B). In contrast, the polyclonal and A2 ΔCAR-eGFP Tregs were expected to exhibit a similar suppressive profile irrespective of the presence of HLA-A2.*

The suppressive profile of the polyclonal Tregs cultured with HLA-A2$^+$ and HLA-A2$^-$ B-LCLs was identical (Figure 5-5A). This indicated that, as expected, the presence of HLA-A2 did not influence the suppressive capacity of these cells. In contrast, A2 CAR-eGFP Tregs inhibited Tresp proliferation more effectively (*p*-values ranging from 0.0082 to 1.1x10^{-5}; n=5) in the presence of HLA-A2$^+$ B-LCLs, compared to HLA-A2$^-$ B-LCLs, at Treg:Tresp ratios ranging from 1:2 to 1:64 (Figure 5-5B). At a 1:32 Treg:Tresp ratio, A2 CAR-eGFP Tregs inhibited Tresp proliferation by 62.8 ± 5.2% in the presence of HLA-A2$^+$ B-LCLs but only 35.8 ± 3.5% in the presence of HLA-A2$^-$ B-LCLs. Similarly, at a 1:64 Treg:Tresp ratio, Tresp proliferation was inhibited in the presence of HLA-A2$^+$ B-LCLs by 38.1 ± 3.6% and in the presence of HLA-A2$^-$ B-LCLs by 16.9 ± 3.4%. Overall, these results suggested that at physiologically-relevant Treg:Tresp ratios, A2 CAR-eGFP Tregs were twice as potent as polyclonal Tregs at suppressing Tresp proliferation in the presence of HLA-A2$^+$ APCs. Interestingly, the suppressive capacity of the A2 ΔCAR-eGFP Tregs was also influenced by the presence of HLA-A2 (Figure 5-5C); A2 ΔCAR-eGFP Tregs were significantly more

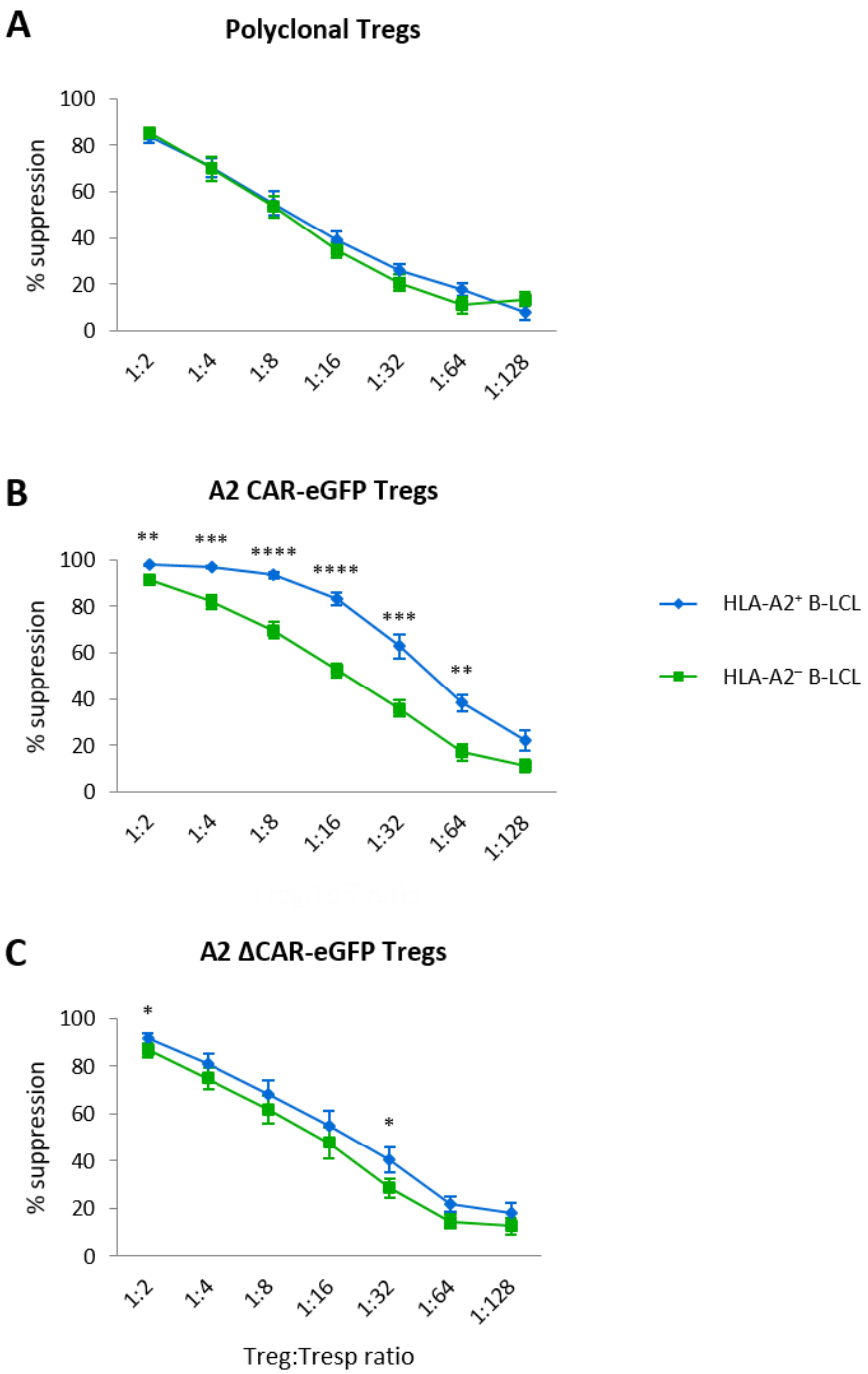

Figure 5-5 | **Human Tregs expressing the A2 CAR-eGFP construct suppressed autologous CD4$^+$CD25$^-$ Tresp proliferation with a greater potency in the presence of HLA-A2$^+$ antigen presenting cells.** *The efficacy with which human Tregs suppressed autologous Tresp proliferation in the presence HLA-A2$^+$ or HLA-A2$^-$ APCs was determined at the end of the Treg expansion period. Polyclonal (A), A2 CAR-eGFP (B) and A2 ΔCAR-eGFP (C) Tregs were co-cultured with 1x10^5 autologous CTV-labelled CD4$^+$CD25$^-$ Tresps and 3x10^4 irradiated (120 Gy) B-LCLs. Assays were performed in parallel where one co-culture contained HLA-A2$^+$ B-LCLs (blue line) and the other co-culture contained HLA-A2$^-$ B-LCLs (green line). In each assay pairing, the B-LCLs used expressed the same HLA-DR molecule (HLA-DR7 or HLA-DR11). Assays were performed in duplicate and the data shown represents mean ± SEM pooled from 5 individual experiments. Significance was determined using a two-tailed paired Student's t-test where *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p0.0001.*

suppressive in the presence of HLA-A2$^+$ B-LCLs compared to HLA-A2$^-$ B-LCLs at Treg:Tresp ratios of 1:2 (p=0.041) and a 1:32 (p=0.027). This minor increase in suppressive ability may have been due to the antigen-targeting moiety of the A2 ΔCAR-eGFP molecule facilitating an interaction between the A2 ΔCAR-eGFP Tregs and the HLA-A2$^+$ APCs, bringing the Treg into the vicinity of the Tresp:B-LCL interaction.

5.3.2.2 A2 CAR-eGFP Tregs did not exert their suppressive capacity in a cytotoxic manner

Studies investigating the mechanisms by which Tregs function have demonstrated that Tregs can elicit cytotoxicity towards target cells including tumour cells (Cao, Cai et al. 2007), autologous T cells and APCs (Grossman, Verbsky et al. 2004) by secreting granzyme and perforin. This mechanism of function was of particular concern as in the envisaged transplant setting, the principle cells which expressed the target antigen (HLA-A2) were the allograft cells. If a cytotoxic response was directed towards HLA-A2$^+$ cells *in vivo*, the A2 CAR-eGFP Tregs would inadvertently, but specifically, target and destroy the cells which comprised the allograft, thereby contributing to allograft rejection instead of preventing it.

To investigate whether A2 CAR-eGFP Tregs elicited a cytotoxic function in a HLA-A2-specific manner, Tregs were co-cultured overnight with MCF-7 (HLA-A2$^+$) and T-47D (HLA-A2$^-$) cell monolayers, as previously described (section 3.3.2.2). CD4$^+$CD25$^+$ Tregs and control CD4$^+$CD25$^-$ Tconvs, were lentivirally transduced to express the A2 CAR constructs, cell sorted based on their eGFP expression, re-stimulated and expanded for an addition 10 days prior to the assays being performed. Following co-culture, the viability of the monolayer cells was measured by MTT assay and supernatants were collected to measured cytokine production. In a similar manner to the A2 CAR-eGFP-expressing unfractioned T cells (Figure 3-19), A2 CAR-eGFP CD4$^+$CD25$^-$ Tconvs specifically destroyed the HLA-A2$^+$ (MCF-7) cell monolayers (Figure 5-6; n=5). In contrast, Tregs expressing this CAR exhibited no detectable level of cytotoxicity towards either the MCF-7 or T-47D (HLA-A2$^-$) cell monolayers (n=3).

Looking at the cytokines which were produced during co-culture, IL-2 was detectable only in the supernatants of A2 CAR-eGFP Tconvs which were co-cultured with MCF-7 cells (139 pg/mL; Figure 5-7A; n=5). Upon co-culture with MCF-7 cells, A2 CAR-eGFP Tconvs produced significantly high levels of the pro-inflammatory cytokine IFNγ (11,210 ± 3,937.0 pg/mL) compared to polyclonal (36.2 ± 10.8 pg/mL) or A2 ΔCAR-eGFP Tconvs (83.3 ± 40.4 pg/mL ;p<0.047) and compared to co-cultures with T-47D cells (26.1 ± 9.0 pg/mL; p=0.047; Figure 5-7B). A2 CAR-eGFP

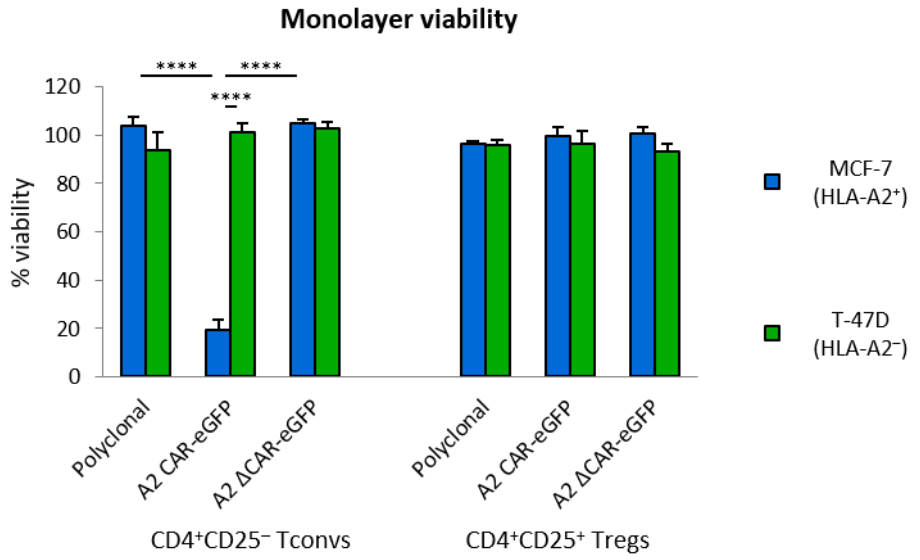

Figure 5-6 | **Human Tregs expressing the A2 CAR-eGFP construct did not elicit cytotoxic activity in a HLA-A2-specific manner.** *Human Tconvs and Tregs were harvested at the end of their expansion period and co-cultured overnight with confluent monolayers of HLA-A2$^+$ MCF-7 cells and HLA-A2$^-$ T-47D cells. The viability of the monolayer cells was measured after co-culture by MTT assay. % viability was calculated relative to monolayer cells cultured alone. Data is mean + SEM pooled from 3-5 individual experiments. Statistical significance was determined using a two-tailed paired Student's t-test where ****=p<0.0001.*

Tregs also produced significant levels of IFNγ upon co-culture with HLA-A2$^+$ cells (323.3 ± 88.6 pg/mL; n=3) although this was 35-fold less than that produced by the A2 CAR-eGFP Tconvs (Figure 5-7B). In contrast, the quantity of the immunosuppressive cytokine IL-10 secreted A2 CAR-eGFP Tregs was 2.7-fold higher than that secreted by A2 CAR-eGFP Tconvs following antigen recognition (Figure 5-7C). Upon culture with MCF-7 cells, A2 CAR-eGFP Tregs produced significantly high levels of the IL-10 (1,162 ± 274 pg/µL) compared to polyclonal Tregs (not detected) and A2 ΔCAR-eGFP Tregs (11.6 ± 1.5 pg/µL). These results demonstrated that compared to CD4$^+$CD25$^-$ Tconvs, Tregs expressing the A2 CAR-eGFP construct preferentially secreted low quantities of the pro-inflammatory cytokine IFNγ and high quantities of the anti-inflammatory cytokine IL-10.

Taken together, these findings suggested that *in vivo*, Tregs expressing the A2 CAR-eGFP construct would not elicit cytotoxicity towards the allograft cells but instead, would likely be activated within the graft and contribute to the establishment of an intragraft immunosuppressive milieu.

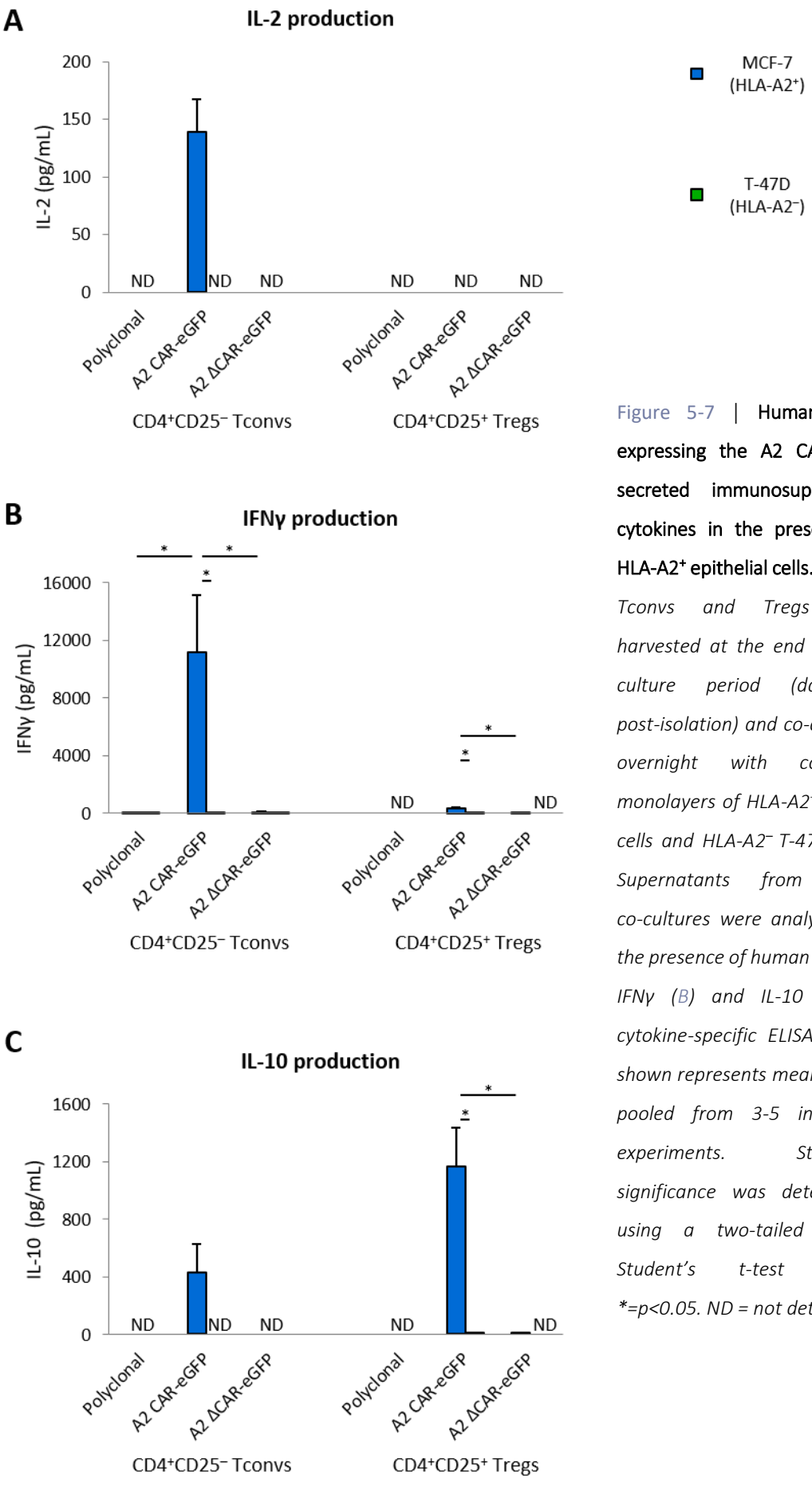

Figure 5-7 | **Human Tregs expressing the A2 CAR-eGFP secreted immunosuppressive cytokines in the presence of HLA-A2$^+$ epithelial cells.** *Human Tconvs and Tregs were harvested at the end of their culture period (day 20 post-isolation) and co-cultured overnight with confluent monolayers of HLA-A2$^+$ MCF-7 cells and HLA-A2$^-$ T-47D cells. Supernatants from these co-cultures were analysed for the presence of human IL-2 (A), IFNγ (B) and IL-10 (C) by cytokine-specific ELISAs. Data shown represents mean + SEM pooled from 3-5 individual experiments. Statistical significance was determined using a two-tailed paired Student's t-test where *=p<0.05. ND = not detected.*

5.3.2.3 Human Tregs expressing a HLA-A2-specific CAR preferentially transmigrated across HLA-A2$^+$ endothelial cell monolayers

One of the key purposes of using a CAR to redirect Tregs towards a donor HLA class I molecule was the fact that unlike HLA class II molecules, HLA class I is ubiquitously expressed in allografts. As such, in addition to assessing the functionality of A2 CAR-eGFP Tregs in the presence of HLA-A2, it was of particular interest to investigate how expression of this CAR influenced the ability of the Tregs to migrate into HLA-A2$^+$ tissues. To evaluate this *in vitro*, a transmigration assay was performed to determine whether Tregs expressing the A2 CAR-eGFP construct preferentially

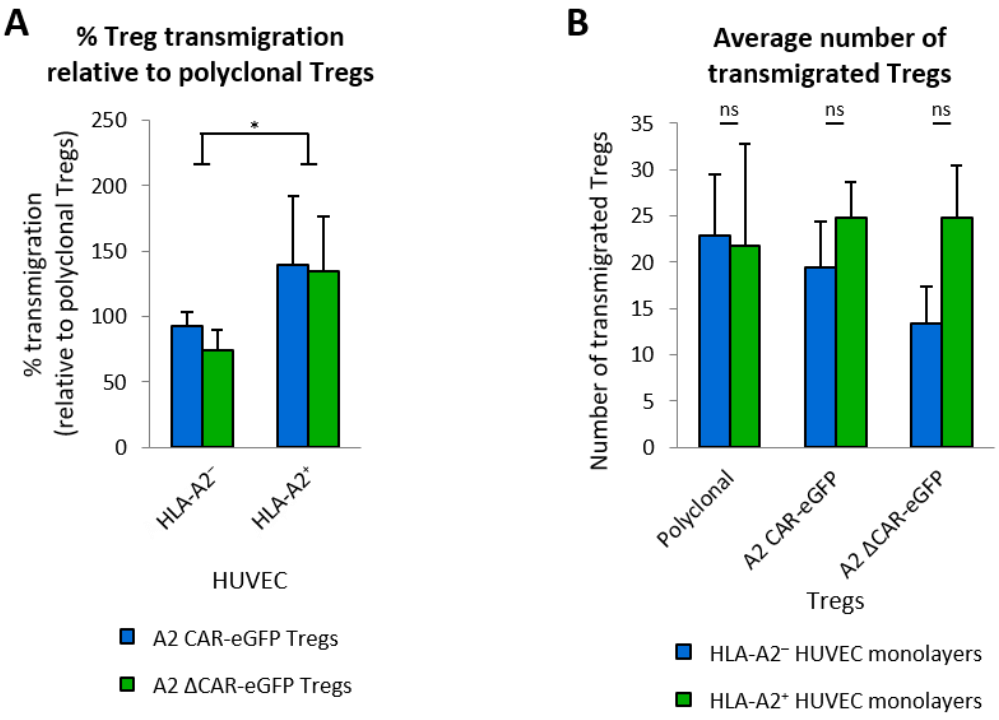

Figure 5-8 | **Human Tregs expressing a HLA-A2-specific CAR preferentially transmigrated across HLA-A2$^+$ endothelial cell monolayers.** *HLA-A2$^+$ and HLA-A2$^-$ HUVEC cell monolayers were pre-treated with IFNγ for 72 hours prior to the assay in order to upregulate HLA class I expression. Human Tregs were harvested at the end of their expansion period and flowed across HUVEC monolayers with a shear stress of 1 dyn/cm^2. The number of Tregs which transmigrated through the monolayers was measured. Data shown represents mean + SEM pooled from 2-6 individual experiments. A: % transmigration was calculated as the number of A2 CAR-eGFP (blue) or A2 ΔCAR-eGFP (green) Tregs which transmigrated relative to the number of polyclonal Tregs which transmigrated. Statistical significance was determined using a two-way ANOVA where *=p<0.05. B: The average number of Tregs which transmigrated through the HLA-A2$^-$ (blue) and HLA-A2$^+$ (green) HUVEC monolayers within the 10 second analysis frames is shown. Statistical significance was determined using a one-tailed unpaired Student's t-test. ns = not significant.*

transmigrated across HLA-A2-expressing endothelial (HUVEC) monolayers, compared to HLA-A2$^-$ HUVEC monolayers.

Polyclonal, A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs were flowed across HLA-A2$^+$ and HLA-A2$^-$ HUVEC monolayers. To mimic inflammatory conditions which would be expected *in vivo*, the HUVECs were pre-treated with IFNγ to upregulate HLA class I expression and the Treg lines were flowed with a shear stress of 1 dyn/cm^2. The Tregs were imaged to measure the rate at which they adhered and transmigrated (Hayhoe, Kamal et al. 2006). Relative to polyclonal Tregs, the A2 CAR-eGFP and the A2 ΔCAR-eGFP Tregs transmigrated through HLA-A2$^+$ endothelial monolayers at a significantly faster rate than HLA-A2$^-$ endothelial monolayers (p=0.042; Figure 5-8A; n=2-6). However, when this data was analysed independently of the polyclonal Tregs, statistical significance was not achieved (Figure 5-8B) although trends were observed for both the A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs to transmigrate faster across HLA-A2$^+$ HUVEC monolayers. Interestingly, in this latter analysis, the preferential transmigration was more evident for the A2 ΔCAR-eGFP Tregs than the A2 CAR-eGFP Tregs, possibly due to the higher level of expression of the A2 ΔCAR-eGFP construct in the Tregs (eGFP MFI in Figure 4-6A). Overall, these results demonstrated that Tregs expressing a HLA-A2-specific CAR preferentially transmigrated through HLA-A2$^+$ HUVECs. Furthermore, as Tregs containing the truncated A2 ΔCAR-eGFP construct which lacked a CD28-CD3ζ signalling domain were able to transmigrate through the HLA-A2$^+$ HUVEC monolayers at a similar rate to Tregs which expressed the full-length A2 CAR-eGFP construct, this result also suggested that the transmigration was driven by the CAR:HLA-A2 ligation process and did not rely on the activation of signalling cascades within the Treg. Overall, this result demonstrated that Tregs expressing a CAR with a HLA-A2-recognition domain preferentially transmigrated across HLA-A2$^+$ HUVEC endothelial monolayers.

5.3.3 Investigation of the efficacy of human A2 CAR Tregs *in vivo*

Having demonstrated the functional superiority and enhanced transmigratory capacity of A2 CAR-eGFP Tregs in the presence of HLA-A2 *in vitro*, the next step was to investigate the protective capacity of A2 CAR-eGFP Tregs in the presence of HLA-A2 *in vivo*.

5.3.3.1 Assessment of the efficacy of A2 CAR-eGFP Tregs using a xeno-GvHD model

The first clinical trials to demonstrate the efficacy of polyclonal Treg therapy were performed in recipients of allogeneic HSCT where the principle aim was to reduce the incidence of GvHD (Trzonkowski, Bieniaszewska et al. 2009, Brunstein, Miller et al. 2011, Di Ianni, Falzetti et al. 2011, Theil, Tuve et al. 2015). This has previously been modelled in humanised mice. One of the most common models currently used is to infuse human PBMCs into immunocompromised mice, allowing xenogeneic activation of the PBMCs and development of xeno-GvHD (Ali, Flutter et al. 2012).

To compare the protective efficacy of A2 CAR-eGFP Tregs and polyclonal Tregs in the presence of HLA-A2 *in vivo*, a xeno-GvHD model was employed using immunodeficient HLA-A2$^+$ transgenic Ch1-2hSa mice (Serra-Hassoun, Bourgine et al. 2014). Additional details relating to these mice are provided in Table 2-1 but briefly, these are mice which lack the expression of murine MHC complexes and instead, present the human HLA complexes HLA-A2 and HLA-DR1. Consequently,

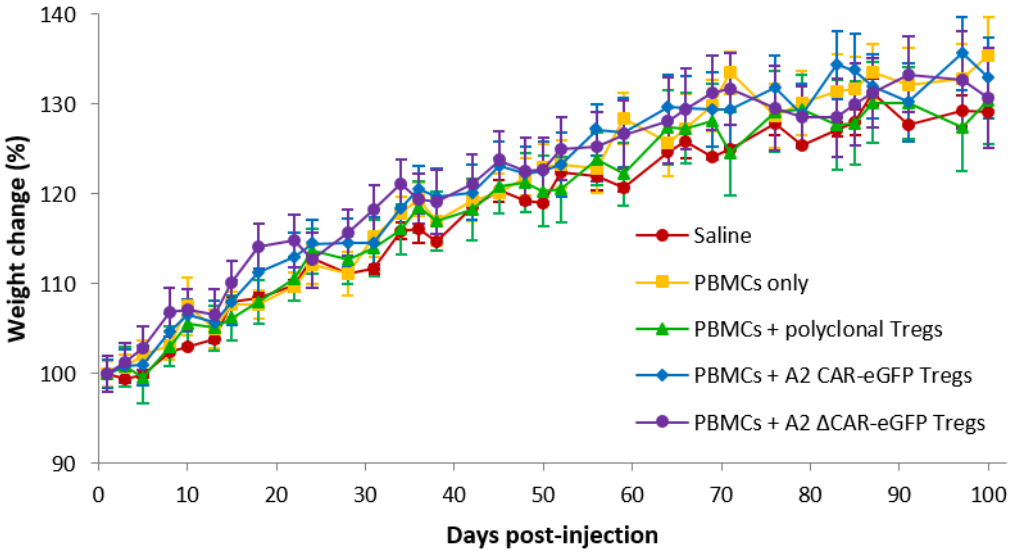

Figure 5-9 | **Immunodeficient HLA-A2$^+$ Ch1-2hSa transgenic mice did not develop xeno-GvHD following iv adoptive transfer of 10^7 allogeneic CD25-depleted PBMCs.** *Ch1-2hSa mice were injected iv with saline (red line) or 1x10^7 allogeneic CD25-depleted PBMCs (orange line) ± 2x10^6 autologous Tregs which were untransduced (polyclonal; green line) or engineered to express the A2 CAR-eGFP (blue line) or A2 ΔCAR-eGFP construct (purple line). Mice were 6-7 weeks old at the time of injection. Mice were monitored for signs of xeno-GvHD including weight loss which was measured every 2-3 days. % weight change was calculated relative to the initial weight of the mice on the day of cell transfer (day 0). Data shown represents mean ± SD with 3-4 mice per group and is representative of 2 individual experiments.*

it was expected that A2 CAR-eGFP Tregs would have an enhanced protective capacity in these mice which ubiquitously express HLA-A2. Ch1-2hSa mice were injected i.v. with $1x10^7$ allogeneic HLA-A2$^-$ CD25$^-$ PBMCs ± $2x10^6$ autologous Tregs and monitored regularly for signs of GvHD but unfortunately, contrary to previous studies (Ali, Flutter et al. 2012), these mice continued to gain weight and ultimately, did not develop GvHD (Figure 5-9). An analysis of leukocytes in the peripheral blood of these mice was performed 1.5 and 2.5 months post-transfer. RBC-depleted leukocytes from the peripheral blood were stained with fluorescently-conjugated antibodies specific for human and mouse CD45 and the proportion of cells expressing these markers was measured by flow cytometry. The results suggested that no human CD45$^+$ cells were present in the mice. This finding was confirmed 3 months post-transfer when the mice were sacrificed and an analysis of human CD45$^+$ cells in the spleens was performed (Supplementary data, Figure S6). This lack of human CD45$^+$ cell engraftment may be attributed to a variety of different causes including an insufficient number of PBMCs transferred, the absence of pre-irradiation of the mice or simply the mouse strain which was used. Overall, due to a lack of human cell engraftment in the HLA-A2$^+$ transgenic mice, no meaningful data was obtained from this model.

5.3.3.2 A2 CAR-eGFP-mediated Treg allorecognition conferred a functional advantage in reducing human skin allograft rejection

Due to the unsuccessful establishment of the xeno-GvHD model described above (section 5.3.3.1), the efficacy of the A2 CAR-eGFP Tregs was investigated *in vivo* using a previously established human skin xenograft transplant model (Racki, Covassin et al. 2010, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). This model was previously employed by the host laboratory to demonstrate that human Tregs with direct allospecificity were superior to polyclonal Tregs at inhibiting alloimmune injury. In these studies, Tregs were generated *ex vivo* by expanding polyclonal Tregs against allogeneic DCs (Sagoo, Ali et al. 2011) or B cell (Putnam, Safinia et al. 2013). An extensive description of this model is provided in section 2.7.2 and Figure 2-4. Briefly, human skin explants were transplanted onto immunodeficient recipient mice (NSG or BRG) and allowed to engraft for 5-6 weeks. Mice were then injected iv with $5x10^6$ human PBMCs ± $1x10^6$ Tregs. After an additional 5-6 weeks, the skin grafts were harvested and analysed histologically for various indicators of damage. Transfer of allogeneic PBMCs alone was shown to cause significant levels of damage to the skin grafts whilst co-transfer of Tregs alleviated this response. However, Tregs which had direct allospecificity were shown to protect the skin grafts more effectively than polyclonal Tregs (Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013).

To investigate whether A2 CAR-eGFP Tregs elicited a similar protective role and prevented alloimmune mediated skin graft injury more effectively than polyclonal Tregs, the same model was employed. Human skin was obtained from healthy donors following routine abdominal angioplasty surgery and HLA-A2 expression was determined by flow cytometry using HLA-A2-specific antibodies to stain cells obtained from collagenase-treated skin explants (Figure 5-10). Immunodeficient BRG mice were transplanted with human HLA-A2^{+} skin allografts which were allowed to engraft for 5-6 weeks. The mice were then injected iv with 5x10^{6} allogeneic HLA-A2^{-} CD25^{-} PBMCs ± 1x10^{6} autologous Tregs which were either unmodified or engineered to express the A2 CAR-eGFP or A2 ΔCAR-eGFP constructs.

Skin grafts were explanted 5 weeks following PBMC transfer and analysed histologically for changes in the skin morphology and immune cell infiltration, indicative of graft rejection (Racki, Covassin et al. 2010, Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). The skin grafts were

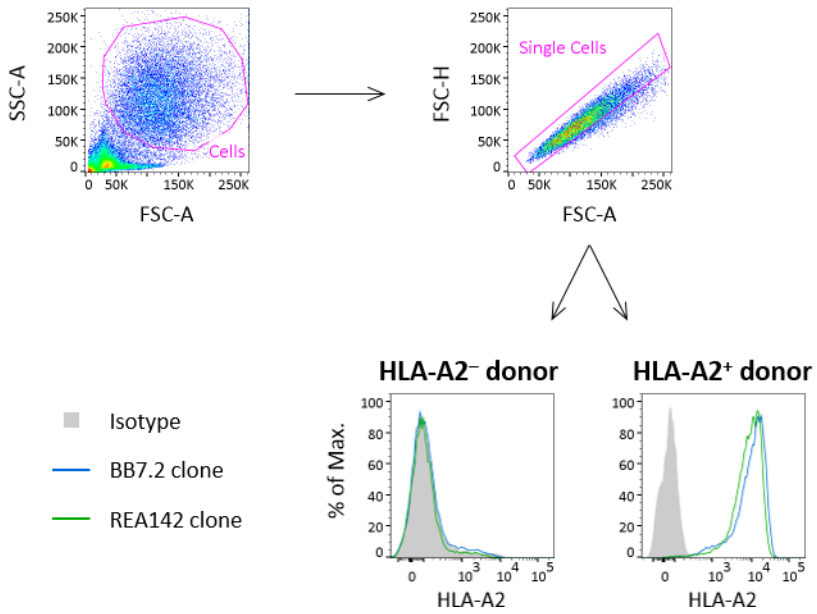

Figure 5-10 | **Skin donor HLA-A2 typing was performed by flow cytometry.** *Healthy human skin was acquired in an anonymised manner from routine abdominal angioplasty procedures and split-thickness (approximately 500 µm) skin sections were generated using a dermatome. To determine whether the skin donor was HLA-A2^{+} or HLA-A2^{-}, a skin section measuring 0.5 cm^{2} was diced as small as possible using scalpels and incubated with 0.5 mg/mL collagenase IV and 0.1 U/mL DNase for 1 hour at 37°C. Cells resulting from this process were passed through a 70 µm cell strainer, washed with FACS buffer (section 2.5.1) and stained with fluorophore-conjugated antibodies specific for HLA-A2. Antibody staining was then assessed by flow cytometry, gating on cell singlets. Two separate antibody clones (BB7.2 represented with blue line and REA142 represented with green line) were used to ensure a false positive result was not acquired from the antibodies cross-reacting with different HLA-A molecules. Control cells were stained with an isotype control (solid grey).*

cryosectioned, fixed and stained using haemotoxalin and eosin (H&E) or antigen-specific antibodies to look for the following indicators of skin damage: increased presence of proliferating $Ki67^+$ keratinocytes, destruction of $CD31^+$ endothelial cells and loss of involucrin, a late stage keratinocyte differentiation marker (Green, Easley et al. 2003). Immune cell infiltration was also assessed by staining sections for the presence of human $CD45^+$, $CD3^+$ and $FOXP3^+$ cells.

A microscopic, non-quantified analysis of the allografts by H&E staining demonstrated that there was dramatically more cell infiltration and damage in the skin graft explants of mice which received PBMCs compared to skin grafts taken from mice which received saline alone (Figure 5-11). Visually, this infiltration and damage appeared to be reduced by the co-transfer of $1x10^6$ Tregs. In the grafts of mice treated with PBMCs, there was a loss of definition between the epidermal and dermal skin layers. This definition was restored by the transfer of Tregs although the thickness of the epidermal layer suggested that general inflammation remained an issue and Treg transfer did not offer complete protection from alloimmune-mediated graft damage.

Immunofluorescence analysis was performed on the allografts to assess the extent of alloimmune-mediated damage in greater detail. Compared to allografts from mice which were injected with saline, significant alloimmune damage was observed in the human skin grafts explanted from mice that received PBMCs only, as demonstrated by a high number of $Ki67^+$ keratinocytes (Figure 5-12A), a loss of $CD31^+$ endothelial cell integrity and loss of the involucrin-expressing epidermal layer (Figure 5-13A). In the skin grafts of mice treated with PBMCs and Tregs, the number of $Ki67^+$ keratinocytes was reduced (Figure 5-12A), $CD31^+$ endothelial cells were protected and an intact involucrin layer was observed (Figure 5-13A). Quantification of these observations was performed and confirmed that whilst treatment with Tregs significantly reduced the number of $Ki67^+$ keratinocytes in the allografts (all $p<0.0005$), A2 CAR-eGFP Tregs achieved this significantly more effectively than polyclonal Tregs ($p=0.042$) where complete protection was offered by the A2 CAR-eGFP Treg treatment (number of $Ki67^+$ keratinocytes/field: saline = 10.9 ± 1.9, PBMC + A2 CAR-eGFP Treg = 10.5 ± 1.3; Figure 5-12B). Interestingly, the A2 ΔCAR-eGFP Tregs also exerted greater protection than the polyclonal Tregs although the extent to which this was achieved did not reach statistical significance ($p=0.11$). Unfortunately, however, the magnitude of these findings was not replicated when the $CD31^+$ blood vessel integrity was analysed as a measure of skin graft damage. In this analysis, no statistical differences were observed between the three Treg lines. However, a trend was observed for the A2 CAR-eGFP Tregs ($CD31^+$ cluster size = 88.9 ± 8.5) to protect the grafts more effectively than the polyclonal (73.3 ± 8.7) or A2 ΔCAR-eGFP (70.9 ± 10.1) Tregs (Figure 5-13B). Overall, these results demonstrated that A2 CAR-eGFP Tregs were more effective than polyclonal Tregs at protecting human skin grafts *in vivo*.

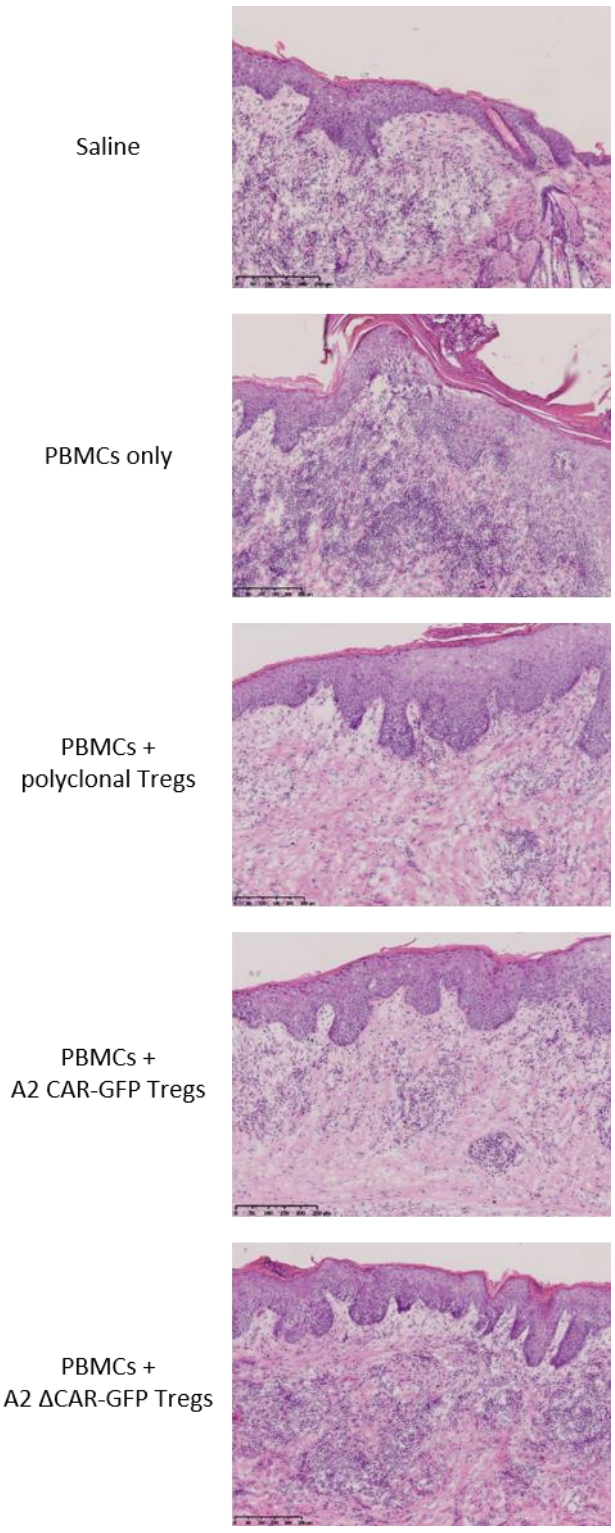

Figure 5-11 | **High levels of cellular infiltration were observed in HLA-A2$^+$ human skin allografts of mice which received 5x10^6 allogeneic PBMCs.** *Immunodeficient BRG mice were transplanted with HLA-A2$^+$ human skin allografts which were allowed to engraft for 6 weeks. Mice were then injected with 5x10^6 allogeneic CD25-depleted PBMCs ± 1x10^6 autologous Tregs which were untransduced (polyclonal) or engineered to express the A2 CAR-eGFP or A2 ΔCAR-eGFP construct. The mice were sacrificed 6 weeks post-transfer at which point the allografts were harvested and cryopreserved. 8 µm cryosections were stained with H&E and analysed by microscopy. Images shown are representative of 2-3 mice per group in an experiment which is representative of 2 individual experiments. Scale bars indicate 250 µm.*

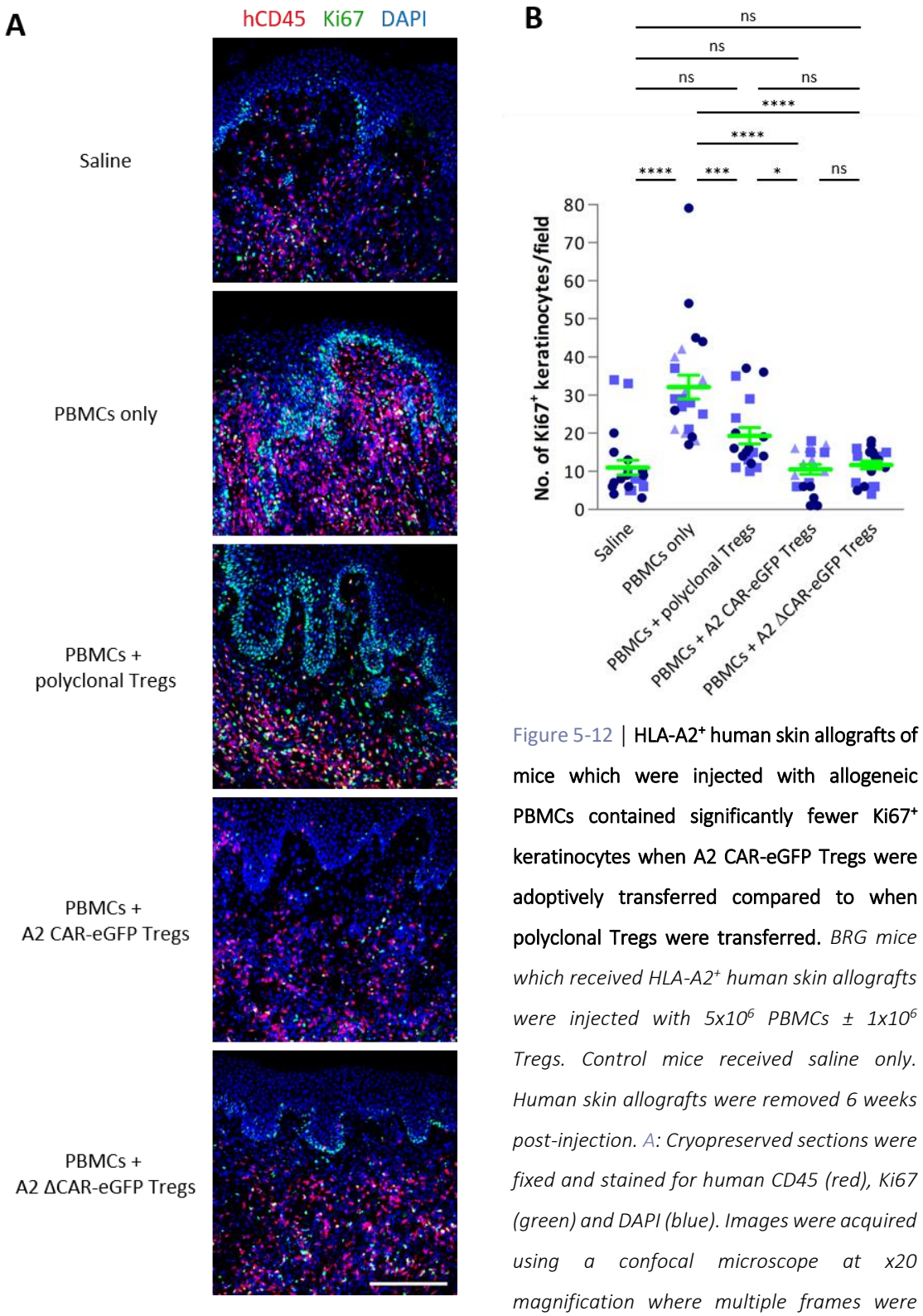

Figure 5-12 | **HLA-A2$^+$ human skin allografts of mice which were injected with allogeneic PBMCs contained significantly fewer Ki67$^+$ keratinocytes when A2 CAR-eGFP Tregs were adoptively transferred compared to when polyclonal Tregs were transferred.** *BRG mice which received HLA-A2$^+$ human skin allografts were injected with 5x10^6 PBMCs ± 1x10^6 Tregs. Control mice received saline only. Human skin allografts were removed 6 weeks post-injection. A: Cryopreserved sections were fixed and stained for human CD45 (red), Ki67 (green) and DAPI (blue). Images were acquired using a confocal microscope at x20 magnification where multiple frames were stitched together using NIS imaging software. Scale bar represents 200 µm. B: The number of Ki67$^+$ proliferating keratinocytes per field was quantified. Explanted grafts were cut into multiple sections and imaged individually by confocal microscopy. 4-6 fields of a fixed size were randomly positioned along the epithelial layer of each confocal acquired image and the total number of Ki67$^+$ cells in each field was counted. Data is pooled from 2-3 mice per group where the contributing points from each mouse are shown in different colours. Data is mean ± SEM and represents 1 of 2 individual experiments. Statistical significance was determined using a one-way ANOVA with a Tukey multiple comparison post-hoc test where *=p<0.05, ***=p<0.001 and ****=p<0.0001. ns = not significant.*

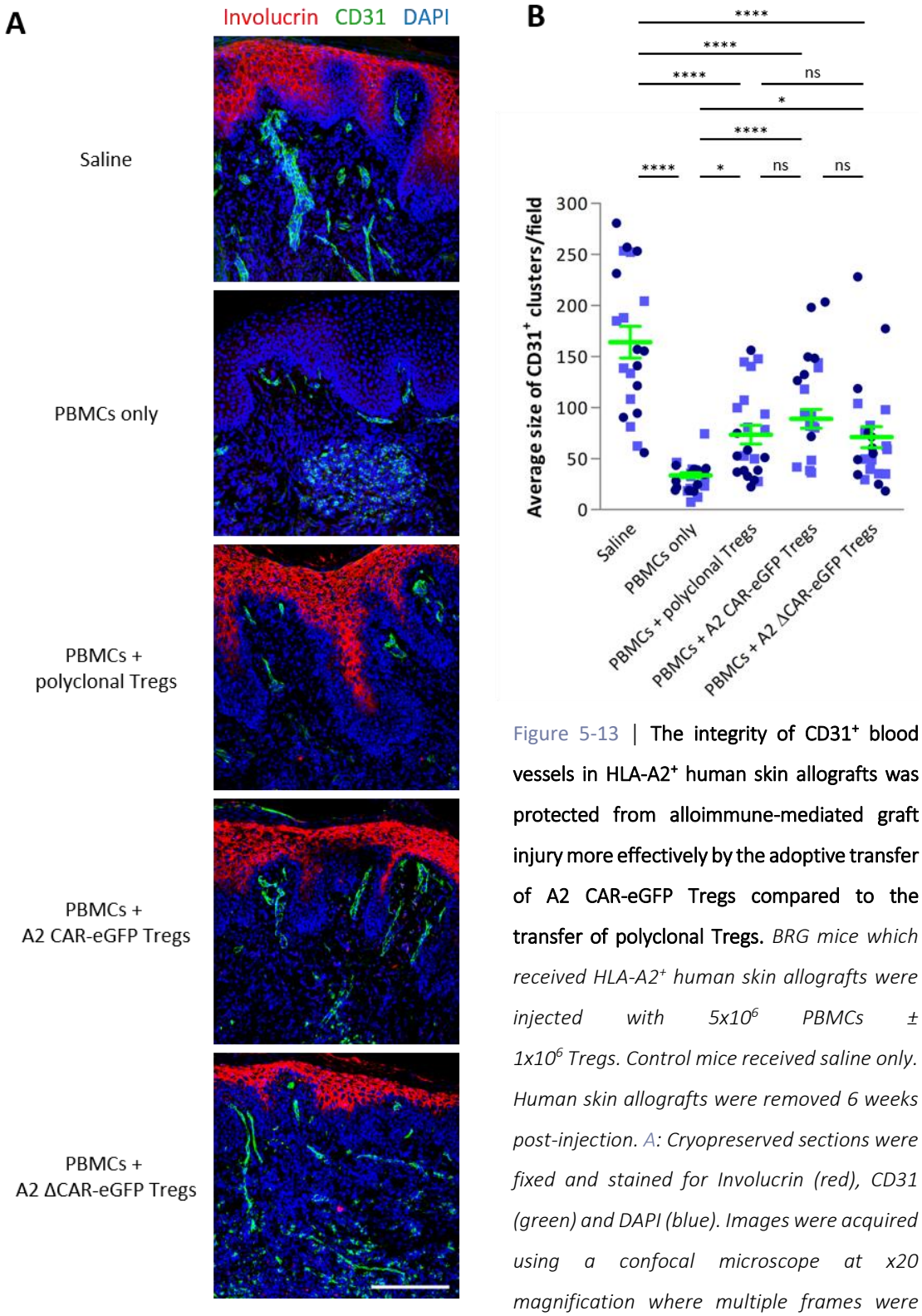

Figure 5-13 | **The integrity of CD31⁺ blood vessels in HLA-A2⁺ human skin allografts was protected from alloimmune-mediated graft injury more effectively by the adoptive transfer of A2 CAR-eGFP Tregs compared to the transfer of polyclonal Tregs.** *BRG mice which received HLA-A2⁺ human skin allografts were injected with 5x10⁶ PBMCs ± 1x10⁶ Tregs. Control mice received saline only. Human skin allografts were removed 6 weeks post-injection. A: Cryopreserved sections were fixed and stained for Involucrin (red), CD31 (green) and DAPI (blue). Images were acquired using a confocal microscope at x20 magnification where multiple frames were stitched together using NIS imaging software. Scale bar represents 200 µm. B: The average size of the CD31⁺ blood vessels per field was quantified. Explanted grafts were cut into multiple sections and imaged individually by confocal microscopy. 4 fields of a fixed size were randomly positioned in the dermal layer of each confocal acquired image and the average size of the blood vessels in each field was calculated using NIS imaging software. Data is pooled from 2-3 mice per group where the contributing points from each mouse are shown in different colours. Data is mean ± SEM and represents 1 of 2 individual experiments. Statistical significance was determined using a one-way ANOVA with a Tukey multiple comparison post-hoc test where *=p<0.05 and ****=p<0.0001. ns = not significant.*

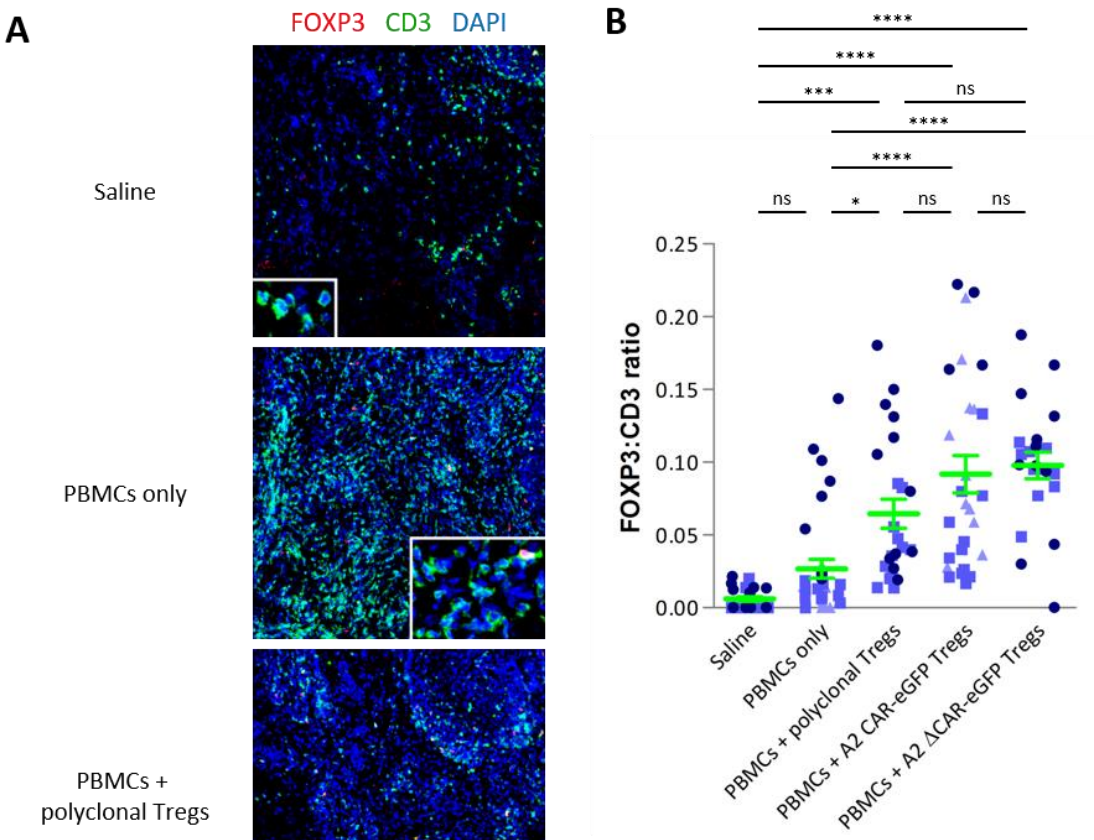

Figure 5-14 | **HLA-A2⁺ human skin allografts of mice which received A2 CAR-eGFP or A2 ΔCAR-eGFP Tregs contained a higher proportion of FOXP3⁺ cells than allografts of mice injected with polyclonal Tregs.** *BRG mice which received HLA-A2⁺ human skin allografts were injected with 5x10⁶ PBMCs ± 1x10⁶ Tregs. Control mice received saline only. Human skin allografts were removed 5 weeks post-injection. A: Cryopreserved sections were fixed and stained for FOXP3 (red), CD3 (green) and DAPI (blue). Images were acquired using a confocal microscope at x20 magnification where multiple frames were stitched together using NIS imaging software. Scale bar represents 200 µm. B: The number of CD3⁺FOXP3⁻ and CD3⁺FOXP3⁺ cells per field was quantified. Explanted grafts were cut into multiple sections and imaged individually by confocal microscopy. 4-6 fields of a fixed size were randomly positioned in the dermal layer of each confocal acquired image and the number of CD3⁺FOXP3⁻ and CD3⁺FOXP3⁺ cells in each field was counted. The number of CD3⁺FOXP3⁺ cells was divided by the number of CD3⁺FOXP3⁻ cells to calculate the FOXP3:CD3 ratio in each field. Data is pooled from 2-3 mice per group where the contributing points from each mouse are shown in different colours. Data is mean ± SEM and represents 1 of 2 individual experiments. Statistical significance was determined using a one-way ANOVA with a Tukey multiple comparison post-hoc test where *=p<0.05, ***=p<0.001 and ****=p<0.0001. ns = not significant.*

An analysis of the immune cell infiltration into the grafted tissue demonstrated that mice which received PBMCs in combination with Tregs had a significantly greater FOXP3:CD3 ratio than mice which received PBMCs alone (Figure 5-14B). This difference in FOXP3:CD3 ratio was more statistically significant when A2 CAR-eGFP (p<0.0001) or A2 ΔCAR-eGFP (p<0.0001) Tregs were transferred, compared to when polyclonal Tregs (p=0.019) were transferred. Furthermore, a trend was observed for the FOXP3:CD3 ratio to be higher in the grafts of mice which received A2 CAR-eGFP or A2 ΔCAR-eGFP Tregs, compared to polyclonal Tregs. Importantly however, no statistically significant differences were observed when the three Treg treatment conditions were directly compared. These findings suggested that the A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs were present at a higher proportion in the HLA-A2$^+$ grafts than the polyclonal Tregs, insinuating a preferential migration into HLA-A2$^+$ tissues (correlating with the findings described in section 5.3.2.3) and/or a preferential retention of these cells in the HLA-A2$^+$ grafts.

In summary, Tregs engineered to express the A2 CAR-eGFP construct inhibited alloimmune mediated injury against HLA-A2$^+$ human skin allografts more effectively than polyclonal or A2 ΔCAR-eGFP Tregs. These results demonstrated the increased potency of A2 CAR-eGFP Tregs compared to polyclonal Tregs *in vivo* and the requirement for the activation of intracellular Treg signalling cascades in order to elicit this response.

5.4 DISCUSSION

In this chapter, A2 CAR-eGFP Tregs were shown to be specifically activated in the presence of HLA-A2, a response which was not replicated by polyclonal or A2 ΔCAR-eGFP Tregs. A2 CAR-eGFP Tregs suppressed autologous CD4$^+$CD25$^-$ Tresp proliferation significantly more effectively in the presence of HLA-A2$^+$ APCs compared to HLA-A2$^-$ APCs which was also not observed for polyclonal or A2 ΔCAR-eGFP Tregs. Furthermore, in the presence of HLA-A2$^+$ target cells, A2 CAR-eGFP Tregs did not exhibit cytotoxicity, unlike their CD4$^+$CD25$^-$ A2 CAR-eGFP Tconv counterparts. Instead, these A2 CAR-eGFP Tregs secreted high levels of the immunoregulatory cytokine IL-10 in the presence of HLA-A2$^+$ target cells. With regards to trafficking, A2 CAR-eGFP Tregs were shown to preferentially transmigrate across HLA-A2$^+$ HUVEC cell monolayers. Interestingly, a similar transmigratory profile was observed for A2 ΔCAR-eGFP Tregs, demonstrating that this process relied on the engagement of HLA-A2 and not on the ability of the CAR to deliver intracellular activatory signals. Using a human skin xenograft transplant model, A2 CAR-eGFP Tregs were shown to protect HLA-A2$^+$ skin allografts from alloimmune-mediated injury more effectively than

polyclonal or A2 ΔCAR-eGFP Tregs, demonstrating the superior protective capacity of these cells *in vivo*.

All previous studies which have investigated the protective capacity of CAR-expressing Tregs have demonstrated the ability of these cells to suppress Tresp proliferation more effectively than polyclonal Tregs when in the presence of their target antigen (Elinav, Waks et al. 2008, Elinav, Adam et al. 2009, Blat, Zigmond et al. 2014, MacDonald, Hoeppli et al. 2016). This was most recently achieved by MacDonald *et al*. who investigated the efficacy of human CAR Tregs which were also redirected towards HLA-A2 (MacDonald, Hoeppli et al. 2016). The A2 CAR generated by these authors incorporated a hybridoma-derived HLA-A2 scFv (BB7.2 clone) which was coupled to a cMyc tag-containing CD28 hinge/transmembrane domain and an intracellular CD28-CD3ζ signalling domain. As such, the composition of this CAR was very similar to that which was generated in Chapter III and only differed in the antigen-targeting ectodomain.

To assess the functionality of their A2 CAR *in vitro*, MacDonald *et al*. performed an antigen-specific suppression assay in which $CD8^+$ T cells were used as Tresps and HLA-$A2^+$ monocyte-derived DCs were used as APC. Their results demonstrated that the A2 CAR Tregs were able to inhibit $CD8^+$ Tresp proliferation but this was achieved with a relatively low efficacy. Their A2 CAR Tregs suppressed Tresp proliferation significantly more effectively than irrelevant CAR Tregs at a 1:5 Treg:Tresp but when lower ratios of 1:10 or 1:20 were analysed, statistical significance was not reached. In contrast, the experiments performed in this chapter used $CD4^+$ T cells as Tresps and demonstrated that the A2 CAR-eGFP Tregs were significantly more suppressive in the presence of HLA-$A2^+$ APCs at Treg:Tresp ratios as low as 1:64, although signalling through the TCR was shown to contribute to this result as polyclonal Tregs also exhibited suppression. Overall, this implied that either the full potential of the A2 CAR Tregs was not demonstrated in the experiments performed by MacDonald *et al*., or that the HLA-A2-specific CAR Tregs generated in this thesis (Chapters III and IV) were more efficacious than those generated by MacDonald *et al*. These authors proceeded to demonstrate that A2 CAR Tregs did not elicit cytotoxicity towards HLA-$A2^+$ K562s, mirroring results described in this chapter.

In cancer research, CAR T cells have been shown to be particularly effective at treating haematological conditions (Davila, Riviere et al. 2014, Maude, Frey et al. 2014). However, these successes have not been translated to the treatment of solid tumours (Dai, Wang et al. 2016). This has been partially attributed to the fact that tumours have an abnormal vasculature (Herman, Savage et al. 2011) which complicates the trafficking of CAR T cells into the tumour. Consequently, various studies in cancer research are investigating strategies which may be employed to improve the trafficking of CAR T cells into the tumour site (Newick, Moon et al. 2016, Newick, O'Brien et

al. 2016). With regards to CAR Tregs, although studies have demonstrated that these cells are present in tissues where their cognate antigen is expressed, (Fransson, Piras et al. 2012, Elinav, Waks et al. 2008, Blat, Zigmond et al. 2014) (discussed in section 1.3.4), no study has specifically investigated the migratory capacity of CAR Tregs *in vitro* or *in vivo*. The results in this chapter demonstrated that A2 CAR-eGFP Tregs preferentially transmigrated through endothelial HUVEC monolayers which expressed HLA-A2. Furthermore, this favoured transmigration was primarily mediated in a Treg activation-independent manner as Tregs expressing the A2 ΔCAR-eGFP construct which lacked an intracellular CD28-CD3ζ signalling domain migrated at a similar rate to Tregs expressing the A2 CAR-eGFP construct. However, the applicability of this finding to cancer research is limited as contrary to transplanted organs and previously mentioned, tumours have an abnormal vasculature and can manipulate local endothelial cells to up/downregulate specific molecules as a mechanism of immune recognition evasion (Buckanovich, Facciabene et al. 2008, Young 2012).

The first approach which was employed to compare the efficacy of polyclonal and A2 CAR-eGFP Tregs *in vivo* in this chapter was a xeno-GvHD model. However, these experiments were unsuccessful as the transferred human cells failed to engraft in the HLA-A2 transgenic mice. The Ch1-2hSa mice used for this model were particularly complicated in their genetic composition in that they not only expressed human HLA genes (HLA-A2 and HLA-DR1), but they also lacked specific genes required for the presentation of murine MHC complexes. This lack of mouse MHC presentation may have severely compromised the engraftment of these mice, as a previous study has demonstrated that human PBMC engraftment is significantly decreased in NSG mice when the *β$_2$-microglobulin* or *I-Aβ^b* genes are removed (King, Covassin et al. 2009). Although this study did not proceed to investigate the effects of knocking out both genes together, as was done when generating the Ch1-2hSa mice, one may assume that the cumulative effect would be even more detrimental for the engraftment of these cells. It was hypothesised that the presence of the human HLA genes would circumvent this issue and provide a more translationally-applicable result but unfortunately, this was not the case, potentially due to the lower immunogenicity of the allo-response compared to the xeno-response. It should also be noted that no previous study has attempted to induce xeno-GvHD in Ch1-2hSa mice and that they have only ever been used to investigate the concept of generating humanised mice with human T cells which are educated against human HLA complexes (Serra-Hassoun, Bourgine et al. 2014). It was possible that the engraftment issues with the Ch1-2hSa mice could have solved by irradiating the mice (van Rijn, Simonetti et al. 2003) or transferring more cells (Ito, Katano et al. 2009). For example, in the study performed by MacDonald *et al.*, recipient NSG mice, which have a proven track record in xeno-GvHD models (Ali, Flutter et al. 2012), were irradiated prior to cell transfer although the

number of PBMCs transferred ($1x10^7$) was the same as the experiment performed in this chapter (MacDonald, Hoeppli et al. 2016). In this study, xeno-GvHD onset was evident from 10 days post-transfer with severe weight loss amongst other factors being observed.

CAR Tregs have been shown to be superior to polyclonal Tregs at protecting various tissues which express a target cognate antigen including the colon (Elinav, Waks et al. 2008, Elinav, Adam et al. 2009, Blat, Zigmond et al. 2014) and CNS (Fransson, Piras et al. 2012). As such, it was reasonable to hypothesise that A2 CAR-eGFP Tregs would protect HLA-$A2^+$ human skin grafts more effectively than polyclonal Tregs. The results acquired from these experiments proved this hypothesis and were comparable to the results of Sagoo *et al.* and Putnam/Safinia *et al*. who demonstrated the superior efficacy of Tregs with direct allospecificity over polyclonal Tregs (Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). In these studies, graft-specific Tregs were generated by stimulating polyclonal Tregs with allogeneic DCs or B cells, respectively. The results in this chapter suggested that the A2 CAR-eGFP Tregs protected human skin grafts with a similar efficacy to the direct allospecific Tregs generated in these studies. A2 CAR-eGFP Tregs were significantly more effective than polyclonal Tregs at reducing the proliferation of keratinocytes (Figure 5-12) in a similar manner to the results of both Sagoo *et al*. and Putnam/Safinia *et al*. Additionally, there was a trend for the A2 CAR-eGFP Tregs and Tregs with direct allospecificity generated by Sagoo *et al*. to protect the integrity of $CD31^+$ blood vessels (Figure 5-13), although this protection was not statistically significant in either case.

The analysis of immune cell infiltration into the HLA-$A2^+$ skin grafts demonstrated that the FOXP3:CD3 ratio in the grafts of mice which received A2 CAR-eGFP or A2 ΔCAR-eGFP Tregs was similar. This finding echoed the results of Putnam/Safinia *et al.* and confirmed the aforementioned Treg trafficking results which suggested that Tregs expressing a HLA-A2-specific CAR favoured homing to a HLA-$A2^+$ graft. This favoured homing may have facilitated the A2 ΔCAR-eGFP Tregs to protect the HLA-$A2^+$ skin grafts more effectively than the polyclonal Tregs (keratinocyte Ki67 expression; Figure 5-12), although this protection was not as great as that which was provided by the A2 CAR-eGFP Tregs ($CD31^+$ blood vessel integrity; Figure 5-13). Together, these results suggested that Tregs expressing the A2 ΔCAR-eGFP which lacked an intracellular signalling domain favoured migration into the HLA-$A2^+$ skin grafts. However, more effective protection was offered by Tregs which expressed the full-length A2 CAR-eGFP construct.

5.4.1 SUMMARY

Overall, the results presented in this chapter demonstrated that A2 CAR-eGFP Tregs were specifically activated and more suppressive than polyclonal or A2 ΔCAR-eGFP Tregs in the presence of HLA-A2$^+$ APCs and that this suppression did not rely on the elicitation of cytotoxicity. A2 CAR-eGFP and A2 ΔCAR-eGFP Tregs preferentially transmigrated across HLA-A2$^+$ HUVEC endothelial monolayers *in vitro* and appeared to preferentially localise in HLA-A2$^+$ skin grafts *in vivo*, compared to polyclonal Tregs. This may have contributed to the superior efficacy with which the A2 CAR-eGFP Tregs protected the HLA-A2$^+$ skin grafts from alloimmune-mediated injury compared to polyclonal Tregs.

CHAPTER VI

GENERATION OF MURINE MHC CLASS I-ALLOSPECIFIC REGULATORY T CELLS USING A NOVEL CHIMERIC ANTIGEN RECEPTOR

6.1 INTRODUCTION

6.1.1 MOUSE VERSUS HUMANISED MOUSE MODELS IN TRANSPLANTATION

The translational aspect of humanised mouse models provides a distinct advantage over mouse models for clinically related studies. However, for certain research questions, meaningful data are more simply acquired when the latter is adopted. A prime example is when comparing the efficacy of Tregs with different allospecificities at limiting transplant rejection.

In a transplant context, Tregs with two different allospecificities are of particular interest: those which recognise intact donor MHC-peptide complexes (direct allospecificity) and those which recognise allopeptides presented in the context of self-MHC molecules (indirect allospecificity). As previously discussed (section 1.1.1.1), Tregs with direct allospecificity exist in mice and humans at a disproportionately high precursor frequency. Approximately 1-10% of the cell in a standard T cell pool have direct allospecificity which is orders of magnitude greater than the ~0.01% of T cells with indirect allospecificity (Veerapathran, Pidala et al. 2011). As such, various studies have successfully isolated and expanded Tregs with direct allospecificity and demonstrated that these cells are more potent than polyclonal Tregs at reducing graft rejection (Sagoo, Ali et al. 2011, Putnam, Safinia et al. 2013). Regarding the human skin xenograft transplant models employed in these studies, it should be noted that only Tregs with direct allospecificity were able to be assessed as the lack of human APC engraftment (including B cells) prevented the priming of human T cells or Tregs with indirect allospecificity.

Studies investigating Tregs with indirect allospecificity have also shown that these cells are more effective than polyclonal Tregs at promoting transplant tolerance (Golshayan, Jiang et al. 2007). However, generating Tregs with indirect allospecificity is difficult as a low number of these cells exist in a recipient, prior to a transplant procedure (Veerapathran, Pidala et al. 2011). Furthermore, once isolated, expanding these cells is difficult as autologous APCs must be effectively and efficiently pulsed with an allopeptide in such a way as to displace the naturally presented self-peptide. Failure to achieve this would result in the expansion of Tregs with self-specificity. A previous study has demonstrated that it is possible to preferentially expand human Tregs with indirect allospecificity *ex vivo* but this was achieved with limited success (Jiang, Tsang et al. 2006). DCs were cultured in the presence of GM-CSF for 5-6 days before being pulsed with a HLA-A2-based peptide and used to stimulate freshly-isolated autologous Tregs. After a week of expansion, only 9% of the Tregs had indirect allospecificity, as determined by tetramer staining (Jiang, Tsang et al. 2006). More promising results were obtained when a similar approach

was employed in a mouse setting (Golshayan, Jiang et al. 2007) although this was subsequently superseded by an approach which involved transducing polyclonal Tregs to express specific TCR α and β chain genes (Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009). In these studies, B6 Tregs were transduced to express the Vα11.2 and Vβ13 TCR chain genes which allowed for the recognition of BALB/c MHC class I (K^d)-peptide presented in the context of B6 MHC class II (I-A^b). Combined with the fact that these Tregs could be maintained in culture long-term, this allowed Tregs with indirect allospecificity to be enriched by over 95% in the mouse setting (Tsang, Tanriver et al. 2008).

In addition to comparing the efficacy of Tregs with self-specificity, direct allospecificity and indirect allospecificity, studies performed in a mouse setting have also explored the potential of Tregs with dual direct and indirect allospecificity. These Tregs were generated by expanding B6 Tregs using allogeneic BALB/c DCs to enrich Tregs with direct allospecificity which were then transduced with the aforementioned TCR α and β chain genes to confer indirect allospecificity. The efficacy of these cells to protect against transplant rejection was assess in two different models: i) a skin transplant model where tail skin from B6.K^d mice was transplanted onto B6 mice, and; ii) a fully-mismatched heart transplant model where hearts were transplanted from BALB/c into B6 mice. In both cases, Tregs with direct allospecificity alone were found to be insufficient at protecting the allografts whilst Tregs with dual specificity prolonged the survival of the skin allografts and protected heart allografts from chronic vasculopathy long-term (Tsang, Tanriver et al. 2008). Similar findings were also published by Joffre *et al*. who used similar transplant models although the authors of this publication did not comprehensively demonstrate that their Tregs indeed possessed both direct and indirect allospecificity (Joffre, Santolaria et al. 2008).

6.1.2 PHOENIX ECO TRANSDUCTION SYSTEM

To generate the aforementioned murine Tregs with indirect allospecificity, polyclonal Tregs were transduced with specific TCR α and β chain genes using the Phoenix Eco transduction system. This is a widely used system originally developed by Nolan *et al*. (Stanford University, California, USA).

As discussed in Chapter III (section 3.1.2), the envelope glycoproteins present on the surface of retroviral virions dictate which target cells are infected. In the laboratory, these glycoproteins can be exploited to not only direct the virion towards specific cell types, but also cells of a specific species (Hamilton, Whittaker et al. 2012, Nomaguchi, Fujita et al. 2012). For example, as discussed previously, HAs from most human influenza viruses target α2,6 sialic acid linkages. However, avian-adapted HAs preferentially bind to α2,3 sialic acid linkages (Couceiro, Paulson et

al. 1993, Gagneux, Cheriyan et al. 2003, Hamilton, Whittaker et al. 2012). This species preference was exploited by Nolan *et al*. to develop two Phoenix virus packaging systems: i) an amphotropic system which yields viral particles capable of infecting dividing cells from most mammalian species, and; ii) an ecotropic system which generates viral particles that can only deliver genes into dividing murine or rat cells (Cepko and Pear 2001).

6.2 AIMS AND OBJECTIVES

The aim of this chapter was to generate a B6 Treg line expressing a functional CAR specific for K^d. The rationale behind this was to ultimately compare the efficacy with which self-, direct allo-, indirect allo-, dual- and CAR-based allo-MHC class I-specific Tregs protected from graft rejection. This was of particular interest as the former four Treg lines mentioned are all MHC class II-restricted thus primarily activated by professional APCs such as DCs. In contrast, the CAR Tregs would be redirect to recognise donor MHC class I, an alloantigen which is ubiquitously expressed on tissue parenchyma throughout an allograft rather than solely on APCs.

To accomplish this, a second generation CAR was generated which incorporated an scFv-based K^d-targeting moiety and a mouse CD28-CD3ζ signalling domain. This CAR was delivered into B6 T cells and Tregs using the Phoenix Eco system previously employed by Tsang *et al*. (Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009). The functionality and specificity of this CAR was then assessed *in vitro* using Tconv and Treg functional assays.

6.3 RESULTS

6.3.1 DESIGN AND GENERATION OF A CAR SPECIFIC FOR K^d

6.3.1.1 Design and construction of a K^d-specific CAR

To generate a mouse-based CAR specific for BALB/c MHC class I (K^d), it was necessary to obtain the sequence of a K^d-specific scFv. Unfortunately, following a literature search, no sequence was identified. As such, a K^d-specific hybridoma cell line (SF1-1.1.10 clone) was purchased from the ATCC® and sequenced by GenScript.

The specificity of this antibody clone was confirmed by flow cytometry. Splenocytes from B6 (K^b) and BALB/c (K^d) mice were stained with antibody purified from the hybridoma followed by a secondary fluorescently-conjugated anti-mouse IgG2a antibody. Antibody produced by the K^d-specific hybridoma cell line specifically bound to BALB/c splenocytes but not B6 splenocytes, demonstrating its specificity (Figure 6-1A).

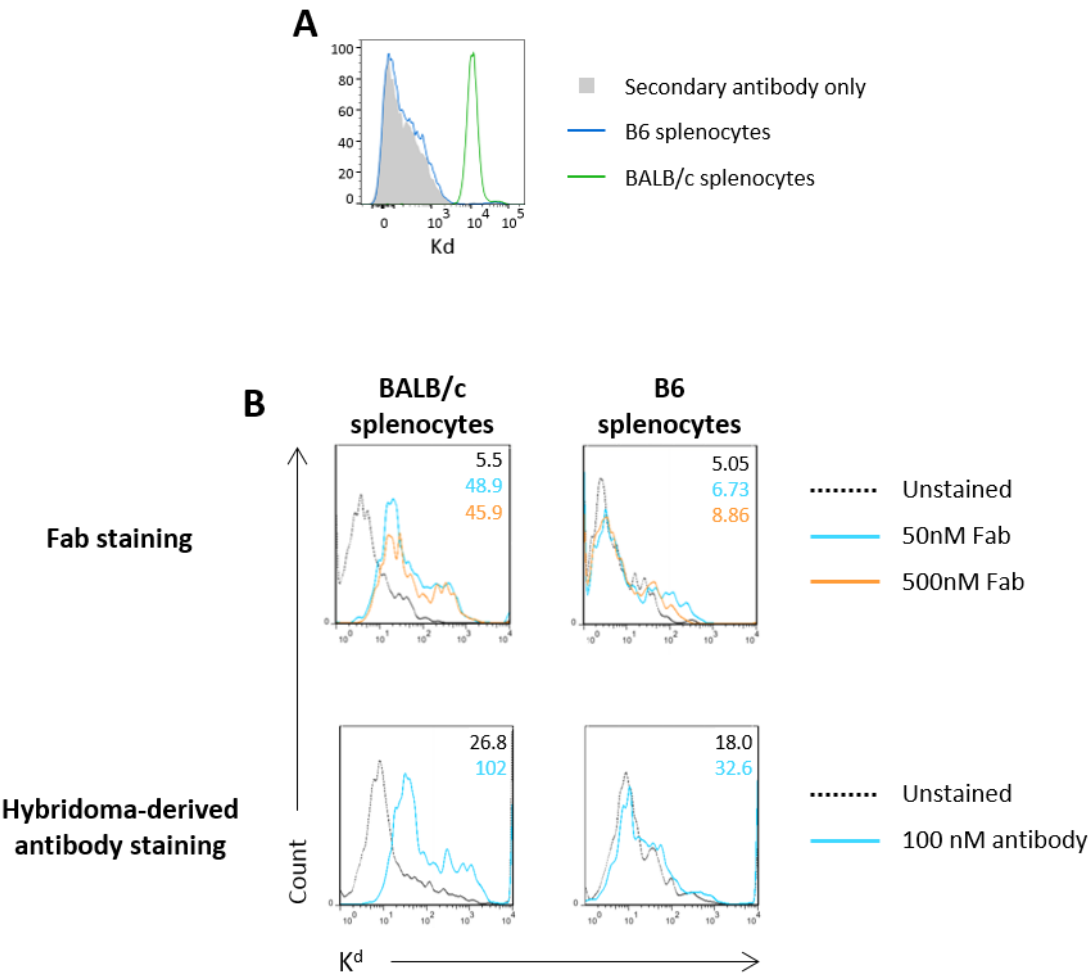

Figure 6-1 | **V_H and V_L chains extracted from the SF1-1.1.10 hybridoma combined to generate a K^d-specific targeting moiety.** *A: An immortalised hybridoma cell line which generated antibody molecules specific for K^d (SF1-1.1.10 clone) was purchased from the ATCC®. Antibody from this hybridoma was used to stain total splenocytes acquired from B6 (blue line) or BALB/c (green line) mice. The binding of this antibody to $CD8^+$ T cells, selected using a fluorescently-conjugated CD8-speicific antibody, is shown. $CD8^+$ T cells stained with the secondary antibody only shown in solid grey. B: The SF1-1.1.10 hybridoma cell line was sequenced by GenScript. Extracted V_H and V_L chain sequences were used to construct a Fab fragment which was used to stained total B6 and BALB/c splenocytes. Binding of the Fab fragment and the original hybridoma-derived antibody (turquoise/orange) is shown, relative to unstained splenocytes (dotted line). MFI values of each sample are provided in the top right corner of each plot.*

A

Nucleotide sequence

GCGGCCGCGGATCCGCCACCATGGCCAGCCCTCTCACCCGGTTTCTGAGCCTCAACCTGCTGT
TGTTGGGTGAGTCCATAATCCTTGGTAGCGGAGAGGCTCAGGTGCAGCTGCAGCAGTCAGGGG
CCGTTCTCGTGAAGCCTGGCGCAAGCGTGAAAATTTCCTGCAAGGTGTCTGGCTACGCTTTTT
CA**ATATACTGGATGAAC**TGGGTCAAGCAGCGACCTGGCAAGGGATTGGAATGGATCGGC**CAGA**
TATTTCCTGGGGACGACGACACCACTTATAATGGAAAGTTCAAGGGAAAGGCTACCCTGACTG
CAGATAAGTCATCAAGTACTGCTTACATGCAGCTCTCGAGCCTGACCAGTGAAGACAGCGCTG
TGTACTTTTGTGCAAGT**GGGCCAATTGGTAAGGGATTTGCCTAC**TGGGGACAGGGCACTCTGG
TGACTGTGTCAGTTAGCTCCGGGGGGGGGTGGATCAGGCGGCGGGGGCAGCGGAGGCGGGGGAA
GTGATGTCCAGATCATCCAGAGCCCCAGCTACCTTGCCGCATCTCCTGGAGAAACAATCATTA
TTAATTGT**AGAGCATCCAAATCCATTAGCCGGTACTTGGCT**TGGTACCAGGAGAAACCTGGTA
AAACAAACAAGCTGCTCATCTAT**TCAGGTAGCACATTGCAGTCC**GGATCTCCCCCCCGCTTTA
GTGGATCTGGGTCCGGAACCGACTTCACCTTAACAATTAGTTCTCTGGAGCCAGAGGATTTCG
CAATGTATTACTGC**CAACAGCATAATGAGTACCCATACACA**TTTGGAGGAGGCACCAAGCTTG
AGATTAAGGTGATCA

B

Amino acid sequence

ATMASPLTRFLSLNLLLLGESIILGSGEAQVQLQQSGAVLVKPGASVKISCKVSGYAFS**IYWM**
NWVKQRPGKGLEWIG**QIFPGDDDTTYNGKFKG**KATLTADKSSSTAYMQLSSLTSEDSAVYFCA
S**GPIGKGFAY**WGQGTLVTVSVSSGGGGSGGGGSGGGGSDVQIIQSPSYLAASPGETIIINC**RA**
SKSISRYLAWYQEKPGKTNKLLIY**SGSTLQS**GSPPRFSGSGSGTDFTLTISSLEPEDFAMYYC
QQHNEYPYTFGGGTKLEIK

GCGGCCGC	NotI restriction site	GGATCC	BamHI restriction site
TGATCA	BclI restriction site	XYZXYZ	V_H chain
XYZXYZ	CD8α leader sequence	XYZXYZ	V_L chain
XYZXYZ	Serine/glycine linker	**XYZXYZ**	Complementarity determining region

Figure 6-2 | **Diagram detailing the components of the K^d-targeting moiety used for constructing the K^d-specific CAR.** *A: Nucleotide sequence of the K^d-targeting moiety. Flanking 5' NotI/BamHI and 3' BclI restriction sites are underlined. B: Amino acid sequence of the K^d-targeting moiety. The sequence of a mouse CD8α leader (red), variable heavy scFv chain (orange), serine/glycine linker (blue) and variable light scFv chain (green) are shown in addition to predicted CDRs in bold.*

Cells from this hybridoma were sent to GenScript for antibody sequencing. Total RNA was extracted from the hybridoma cells and cDNA was synthesised. cDNA corresponding to the V_H and V_L chain genes was then amplified by PCR, cloned into standard vectors and sequenced. Overall, this process provided the genetic sequences of the antibody V_H and V_L chain genes. These extracted sequences were then used by GenScript to design a Fab fragment which was generated in TG1 *E. coli* and purified using an affinity chromatography column approach. The ability of this Fab to bind K^d was then confirmed by GenScript who used a similar flow cytometry approach to that described above whereby B6 and BALB/c splenocytes were stained. The Fab was shown to bind BALB/c splenocytes in a similar manner to the original hybridoma-derived antibody but not

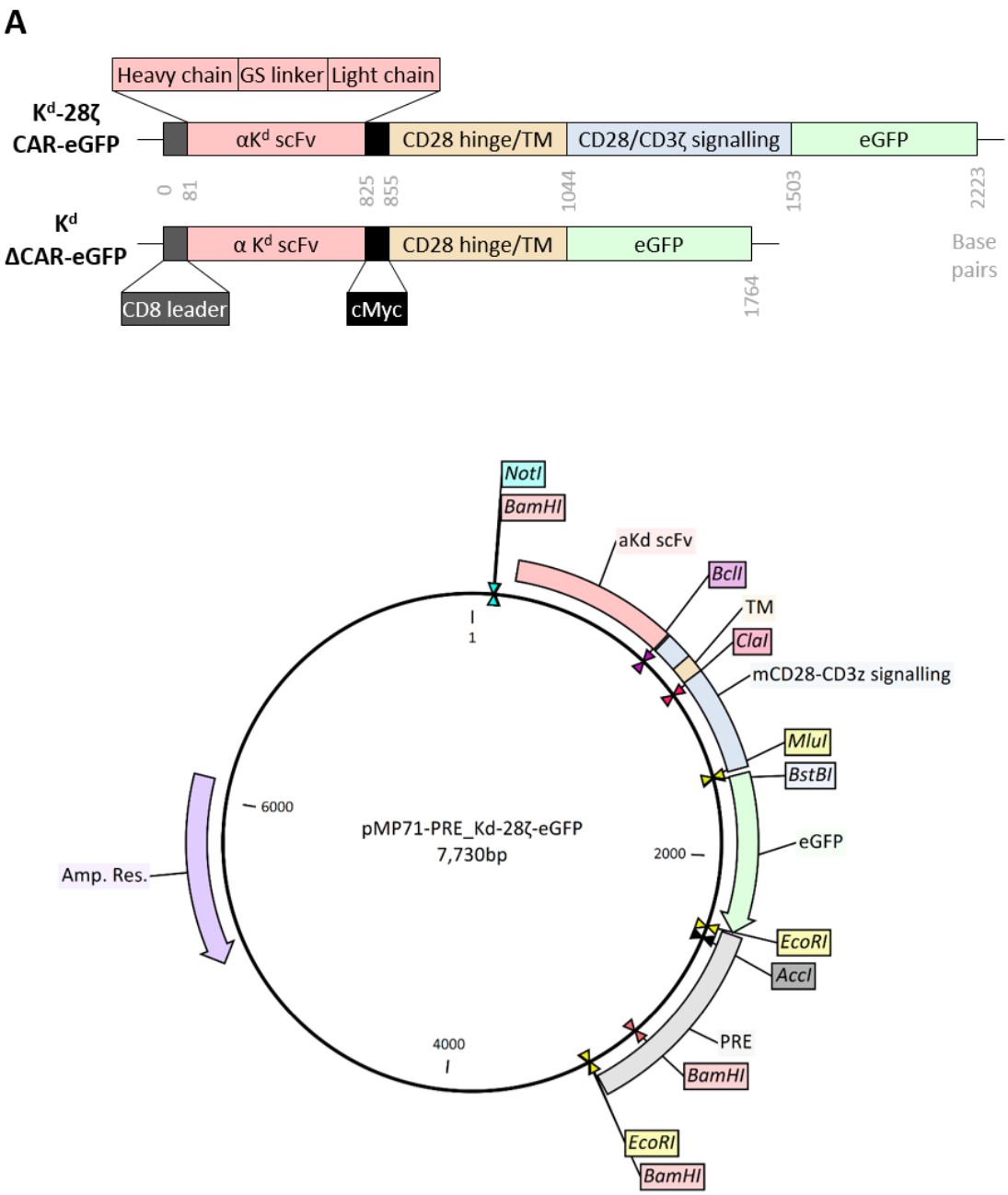

Figure 6-3 | **Schematic diagram of a murine K^d-specific second generation CAR and restriction map of the pMP71-PRE_K^d-28ζ-eGFP retroviral expression vector.** *A: A K^d-specific scFv sequence was designed using the V_H and V_L chain sequences extracted by GenScript (red). A mouse CD8α leader peptide sequence was added upstream of the scFv (grey). The scFv sequence was proceeded by sequences of a mouse CD28 hinge/TM domain (orange) in which the MYPPPY recognition motif was replaced with the 9E10 cMyc epitope (black). Immediately downstream of the CD28 TM domain was either the sequence of a mouse CD28-CD3ζ signalling domain (blue) followed by a direct eGFP fusion (green; K^d-28ζ CAR-eGFP) or the eGFP ORF without the signalling domain present (K^d ΔCAR-eGFP). The complete nucleotide sequence of the K^d CAR-eGFP construct is provided in the Supplementary data, Figure S5. B: Restriction map of the K^d-28ζ-eGFP CAR nucleotide sequence in the pMP71-PRE retroviral expression vector. The CAR gene was gene synthesised by Biomatik and cloned into this vector using the 5' NotI and 3' AccI restriction sites. The complete nucleotide sequence of this vector is provided in the Supplementary data, Figure S6. Amp. Res. = ampicillin resistance gene.*

the B6 splenocytes, confirming that the DNA sequences coded for the V_H and V_L chain sequences of a K^d-specific antibody (Figure 6-1B).

The V_H and V_L chain sequences provided by GenScript were used to design a K^d-specific scFv. This was accomplished by linking the V_H chain sequence to the downstream V_L chain sequence via a $(GGGGS)_3$ serine/glycine linker, as shown in Figure 6-2. The rest of the K^d-specific CAR gene was then designed around this scFv sequence whereby a mouse CD8α leader peptide sequence was added upstream of the scFv sequence to facilitate translocation of the CAR protein to the cell membrane whilst sequences of a murine CD28 transmembrane and CD28-CD3ζ signalling domain were added downstream of the scFv antigen-targeting moiety. Furthermore, to aid downstream analyses, an eGFP ORF was added immediately downstream of the CD28-CD3ζ signalling domain, resulting in the generation of a K^d CAR-eGFP fusion. A schematic of this *in silico* designed gene is provided in Figure 6-3A.

This K^d-28ζ-eGFP CAR nucleotide sequence was gene synthesised by Biomatik (Wilmington, Delaware, USA) and cloned into the pMP71-PRE retroviral vector (kindly provided by Dr Angelika Holler and Prof Hans Stauss; Institute of Immunity and Transplantation, University College London, UK) using the 5' NotI and 3' AccI restriction sites shown in Figure 6-3B. The resulting retroviral expression vector which contained the K^d CAR-eGFP ORF was termed the pMP71-PRE_K^d-28ζ-eGFP construct.

6.3.1.2 Generation of a K^d-specific CAR lacking an intracellular signalling domain

As the K^d-28ζ-eGFP was custom synthesised, it was possible to tailor the gene to incorporate multiple restriction sites where desired. To aid the generation of a truncated K^d CAR lacking a CD28-CD3ζ signalling domain, a ClaI restriction site was incorporated immediately upstream of the first ITAM in the CD28 signalling domain and a BstBI restriction site was added between the end of the CAR ORF, before the eGFP ORF (Figure 6-3B).

A summary of the cloning strategy employed to generate the truncated K^d-specific CAR (K^d ΔCAR-eGFP) is provided in Figure 6-4A. The pMP71-PRE_K^d-28ζ-eGFP vector was digested with ClaI and BstBI to remove the DNA coding for the CD28-CD3ζ signalling domain. Vector DNA containing the truncated CAR gene sequence in a linear form was separated using a 0.6% agarose gel and purified using a membrane-based purification system. Purified DNA was then quantified by running DNA volumes of 1, 3 and 5 µL on a 0.8% agarose gel alongside 5 µL of Hyperladder I. The Hyperladder I bands contained a known concentration of DNA thus the intensity of these

bands could be used to estimate the concentration of purified DNA (Figure 6-5A). The linear DNA of the digested pMP71-PRE vector containing the truncated CAR gene sequence was estimated to have a concentration of 7-8 µg/mL.

As shown in Figure 6-4B, ClaI and BstBI recognise different DNA sequences. However, the sticky ends which remain when these enzymes cleave DNA are the same – both enzymes leave a 5' CG

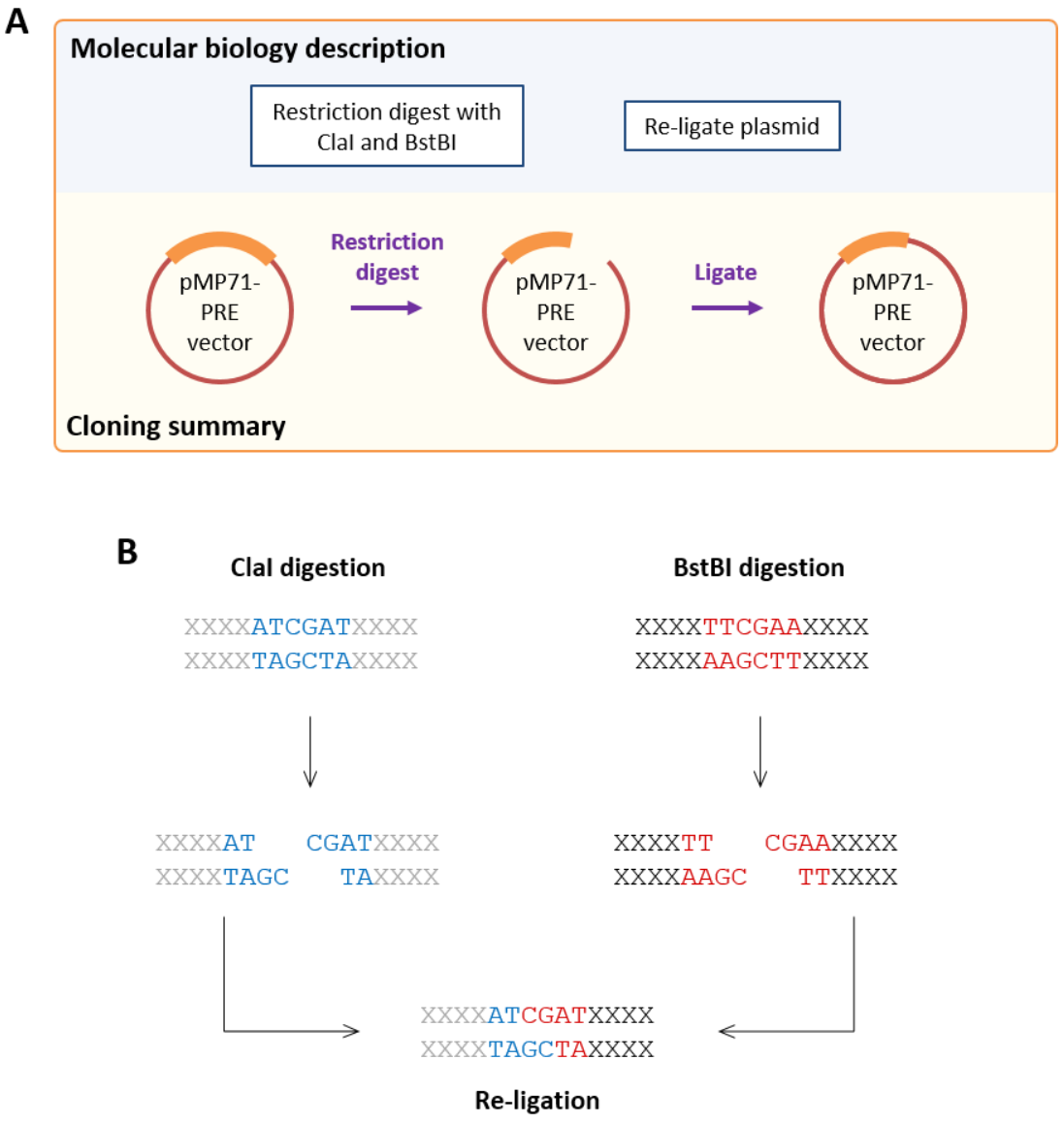

Figure 6-4 | **Cloning strategy for generating a truncated K^d ΔCAR-eGFP construct lacking an intracellular CD28-CD3ζ signalling domain.** *A: Summary of the cloning strategy employed to remove the CD28-CD3ζ signalling domain sequence from the pMP71-PRE_K^d-28ζ-eGFP vector. B: The restriction enzymes ClaI (blue) and BstBI (red) recognise different sequences but both cleave DNA to leave a 5' CG overhang. Consequently, it was possible to re-ligate the DNA products from these digests without an insert. This process abolished the original ClaI and BstBI restriction sites. X = irrelevant nucleotide.*

overhang. As such, it was possible to re-ligate the pMP71-PRE_K^d-eGFP vector directly, negating the requirement to fill in the sticky ends and perform a blunt end ligation. The vector was re-ligated using a T4 ligase which resulted in the generation of a pMP71-PRE vector containing a truncated K^d-eGFP CAR gene (pMP71-PRE_K^d-eGFP or K^d ΔCAR-eGFP).

Products from the ligation were transformed into Stbl3 cells and grown in the presence of ampicillin. Bacterial colonies were harvested, expanded and minipreps were performed to purify DNA. A BamHI restriction digestion was then performed on the isolated DNA to validate the cloning process. This digestion correctly revealed bands of 0.28, 2.4 and 4.5 kbp for the truncated CAR preparations and 0.28, 2.9 and 4.5 kbp for the full-length CAR (Figure 6-5B). Results of this process were confirmed by sequencing prior to downstream application of this construct.

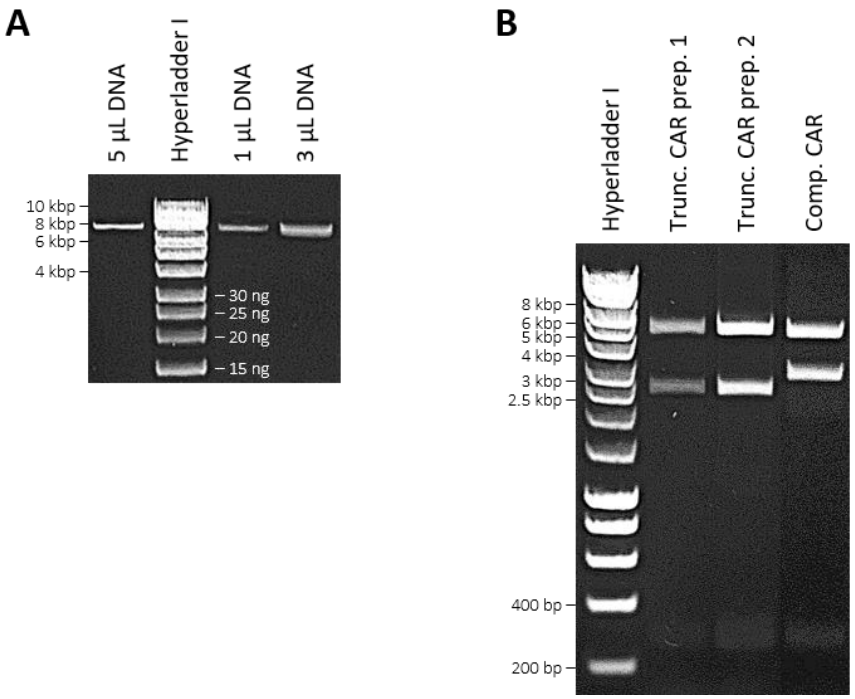

Figure 6-5 | **A truncated K^d-specific CAR was generated by removing the intracellular CD28-CD3ζ signalling domain from the full-length CAR gene sequence.** *A: Quantification of the digested pMP71-PRE vector containing the truncated K^d CAR-eGFP sequence. Desired DNA was isolated on a 0.6% agarose gel and purified using a membrane-based purification system. DNA resulting from this purification was quantified by running 1, 3 and 5 µL of the DNA on a 0.8% agarose gel alongside the 5 µL Hyperladder I. The Hyperladder I bands contained a known quantity of DNA (shown), allowing one to estimate the concentration of a DNA in a sample. This DNA sample was estimated to have a concentration of 7-8 µg/mL. B: BamHI digestion of pMP71-PRE_K^d-eGFP vector minipreps (trunc. CAR) and the pMP71-PRE_K^d-28ζ-eGFP vector (comp. CAR). Resultant fragments were separated on a 0.8% agarose gel which is shown. Expected bands for the full-length CAR digest were 4,600, 2,900 and 300 bp whilst expected bands for the truncated CAR digest were 4,600, 2,400 and 300 bp.*

6.3.2 Characterisation of the K^d-specific CAR using splenocytes

6.3.2.1 Splenocyte-derived T cells were retrovirally transduced to express the K^d CAR-eGFP construct with a high efficiency

The functionality of the novel K^d-specific CAR was initially assessed using murine T cells, employing previously published transduction protocols kindly provided by Dr Angelika Holler and Prof Hans Stauss (Wright, Notley et al. 2009). Retroviral particles containing the pMP71-PRE_K^d-28ζ-eGFP (K^d CAR-eGFP) or pMP71-PRE_K^d-eGFP (K^d ΔCAR-eGFP) vector were generated by transfecting Phoenix Eco cells with the pMP71-PRE vector of interest and a pCL-eco packaging plasmid. High transfection efficiencies with bright eGFP expression were observed in the Phoenix Eco cells by fluorescence microscopy (Figure 6-6). The media of these transfected cells was replaced 24 hours post-transfection such that retroviral particles were released into RPMI-1640 mouse complete cell media (section 2.2.1). This retroviral supernatant was harvested after a further 24 hours and used to transduce splenocytes which had been isolated from B6 mice, activated 24 hours earlier with the mitogen ConA (2µg/mL) and cultured in the presence of 1 ng/mL recombinant human IL-7. Transduced splenocytes were expanded for an additional 3 days prior to analysis and experimentation.

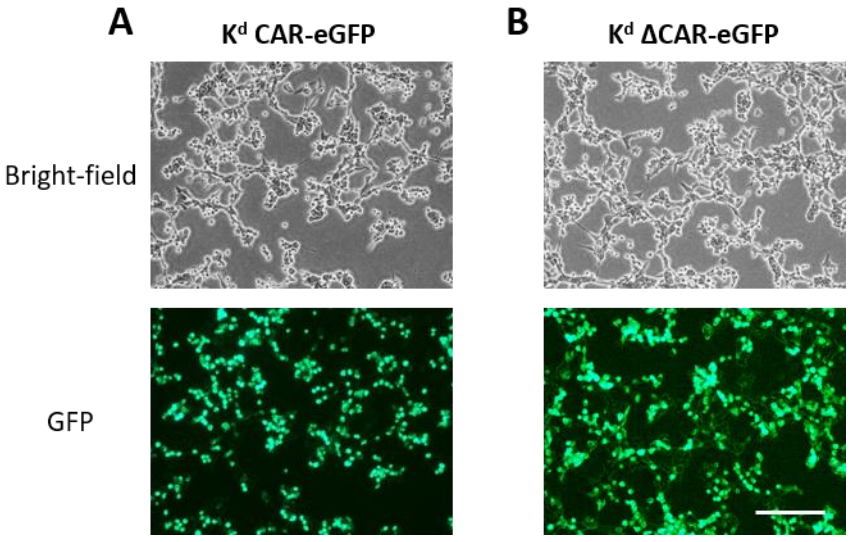

Figure 6-6 | **Fluorescence microscopy images of Phoenix Eco cells transfected to express the K^d CAR-eGFP and K^d ΔCAR-EGFP constructs.** *Phoenix Eco cells were co-transfected to express the pMP71-PRE_K^d-28ζ-eGFP or pMP71-PRE_K^d-eGFP vector and the pCL-eco plasmid, resulting in the generation of retroviral particles. Phoenix Eco cells were visualised by fluorescence microscopy 48 hours post-transfection. Images were taken at 10x magnification. Scale bar represents 200 µm.*

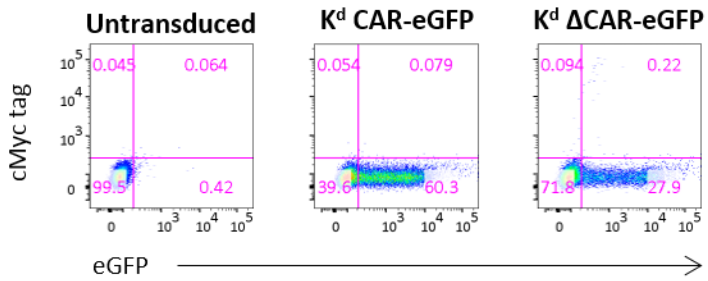

Figure 6-7 | **B6 splenocytes were transduced to express the K^d CAR-eGFP and K^d ΔCAR-eGFP constructs with a high efficiency.** *Total B6 splenocytes were activated with 2 µg/mL ConA and either left untransduced or transduced after 24 hours to express the K^d CAR-eGFP or K^d ΔCAR-eGFP constructs. Cells were expanded for an additional 3 days before being stained with a fluorescently-conjugated antibody specific for the cMyc tag 9E10 epitope. The efficiency of transduction was assessed by flow cytometry, based on eGFP expression. Presence of the CAR on the surface of these cells was determined by cMyc tag staining. Data representative of 4 individual experiments. The percentage of events in each gate is provided.*

Using this protocol, splenocytes were transduced, as measured by eGFP expression, with an average efficiency of 29.4 ± 17.2% (Figure 6-7; n=6). To detect whether the K^d CAR-eGFP construct was present on the cell surface of transduced splenocytes, the cells were stained with a fluorescently-conjugated antibody which was specific for the 9E10 cMyc tag epitope present in the extracellular domain of the CARs (Figure 6-3A). As the eGFP reporter protein was directly fused to the C-terminus of the CARs, the eGFP MFI was directly proportional to the level of CAR expression in these cells. However, despite a high level of eGFP expression in these splenocytes, presence of cMyc tag staining was not detected (Figure 6-7). This suggested that either i) the CAR was not successfully trafficked to the cell surface following synthesis, or; ii) the globular nature of the scFv domain blocked the cMyc tag epitope, preventing binding of the antibody. Overall, this data demonstrated that B6 splenocytes could be transduced to express either the K^d CAR-eGFP and K^d ΔCAR-eGFP constructs.

6.3.2.2 The K^d CAR-eGFP construct appeared to be successfully translocated to the cell surface

To investigate whether the K^d CAR-eGFP protein was successfully trafficked to the cell surface following synthesis, Phoenix Eco cells were seeded onto glass coverslips and transfected to express the K^d CAR-eGFP construct. Cells transfected with the human A2 CAR-eGFP construct

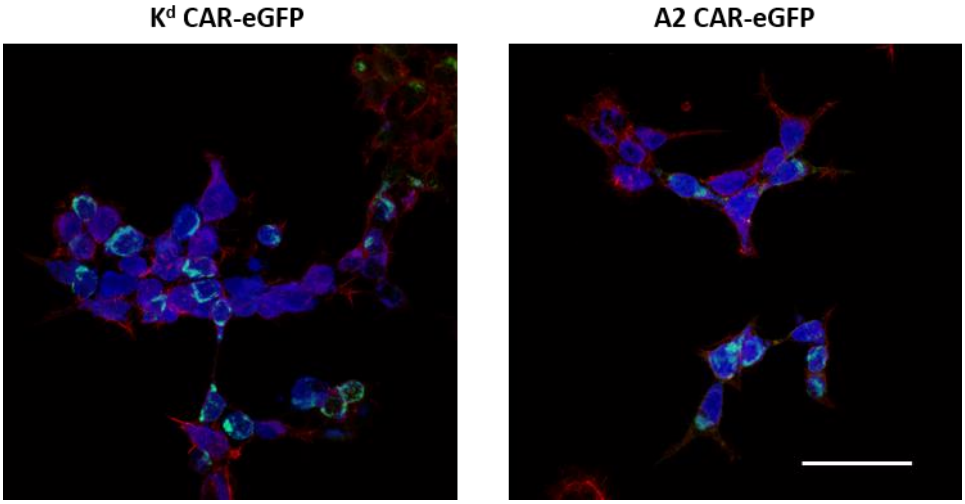

Figure 6-8 | **The K^d CAR-eGFP protein appeared to localise to the cell membrane of Phoenix Eco cells in a similar manner to the A2 CAR-eGFP protein.** *Phoenix Eco cells were seeded onto glass coverslips and transfected to express either the K^d CAR-eGFP protein or the A2 CAR-eGFP protein (green). 24 hours post-transfection, the cells were harvested and stained (in the absence of permeabilisation) with fluorescently-conjugated phalloidin (red), a peptide typically used to label F-actin in cells following permeabilization. This phalloidin staining was performed to distinguish the surface of these cells. The cells were also stained with DAPI (blue) to label the nuclei. 3-4 images per condition were acquired at x60 magnification and data is representative of 2 individual experiments. Scale bar represents 50 µm.*

were used as a positive control. After 24 hours, these cells were stained with fluorescently-conjugated phalloidin (without cell permeabilisation) to label the surface of these cells prior to analysis by confocal microscopy. A comparison of the eGFP localisation in Phoenix Eco cells transfected with the K^d CAR-eGFP construct and the A2 CAR-eGFP construct correlated (Figure 6-8) suggesting that the K^d CAR-eGFP protein may have localised to the cell surface, although this finding was by no means definitive.

6.3.2.3 Splenocytes expressing the K^d CAR-eGFP construct proliferated more when activated by K^d-expressing APCs compared to APCs which did not express K^d

To investigate whether total B6 splenocytes expressing the K^d CAR-eGFP construct preferentially proliferated in the presence of K^d, these cells were co-cultured with APCs isolated from B6, B6.K^d and BALB/c mice. Proliferation was measured after 72 hours of co-culture using two approaches; ^{3}H-thymidine incorporation and CTV dilution. For the former, 1 µCi ^{3}H-thymidine was added to

the cells for the last 18 hours of culture. Alternatively, T cells were labelled with CTV prior to co-culture and the dilution of this dye was used as a measure of proliferation.

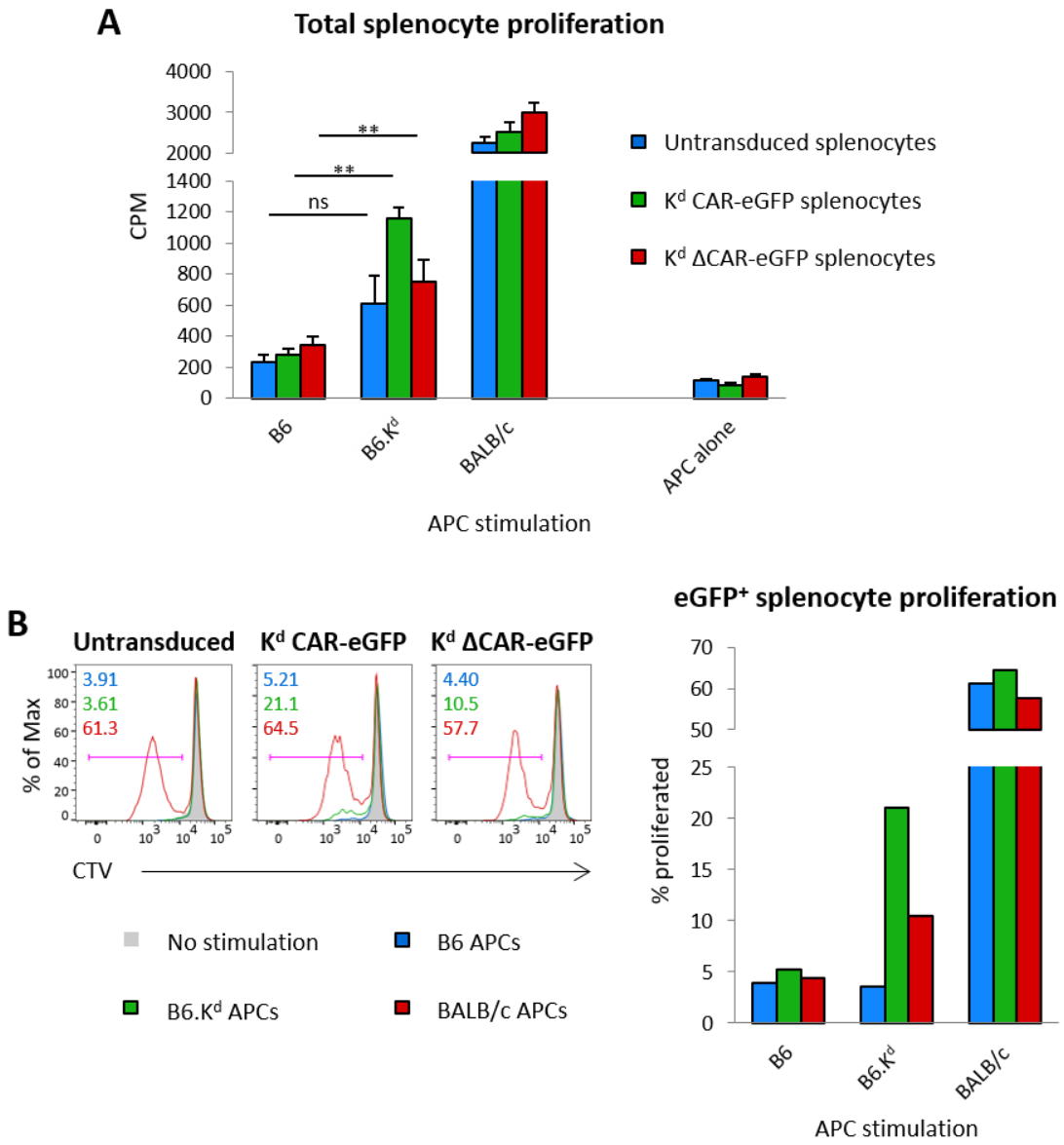

Figure 6-9 | **B6 splenocytes expressing the K^d CAR-eGFP construct preferentially proliferated in the presence of K^d-expressing APCs.** *B6 splenocytes were untransduced or engineered to express the K^d CAR-eGFP or K^d ΔCAR-eGFP construct. These cells were co-cultured with APCs isolated from B6 (blue), B6.K^d (green) or BALB/c (red) mice for 72 hours. Splenocyte proliferation was then measured by ^{3}H-thymidine incorporation (A) or CTV dilution (B). For the ^{3}H-thymidine incorporation (A), co-cultures were pulsed with 1 µCi ^{3}H-thymidine for the last 18 hours of culture and proliferation was quantified using a beta liquid scintillation counting approach. Data is mean + SD of technical triplicates and is representative of 2 individual experiments. Significance was determined using a two-tailed paired Student's t-test where *=p<0.05 and **=p<0.01. cpm = counts per minute. For the CTV-dilution (B), splenocytes were labelled with 2 µM CTV prior to co-culture. Proliferation of only eGFP$^+$ cells was measured, where appropriate and the percentage of cells which divided, as defined by the gate shown, is provided in the top left corner of each plot. Data is mean values only, representing a single preliminary experiment. ns = not significant.*

When proliferation was measured by ^{3}H-thymidine incorporation, expression of K^d by the APC induced a 2.6-fold increase in proliferation of the untransduced T cells, suggesting a high level of background proliferation (*p*=0.069; Figure 6-9A). This may have been due in part to the activation of $CD8^+$ T cells with direct allospecificity against the K^d molecule. The involvement of the direct pathway in activating the T cells was further confirmed when BALB/c APCs were used as stimulators, as the presentation of allogeneic MHC class I (K^d) and MHC class II ($I\text{-}A^d$) by these cells facilitated the activation of both $CD8^+$ and $CD4^+$ T cells with direct allospecificity.

In the same analysis, K^d CAR-eGFP T cells proliferated 4.2-fold more in the presence of B6.K^d APCs, relative to B6 APCs (*p*=0.0049; Figure 6-9A). Given the fact that this was a greater fold-increase than was observed for the untransduced T cells, this suggested that expression of the K^d CAR-eGFP was influencing the proliferation of the T cells. However, the modesty of this difference and the fact that the T cells proliferated 9.3-fold more in the presence of BALB/c APCs draws attention to the low potency of the K^d CAR-eGFP's effect and may even suggest that the CAR was not functioning properly. Interestingly, similar results were also observed for T cells expressing the truncated K^d ΔCAR-eGFP (*p*=0.026), perhaps due to the K^d ΔCAR-eGFP bringing the T cell into the vicinity of the APC, thus promoting the activation of T cells with a low level of direct allospecificity.

Looking solely at the proliferation of the three T cell lines in the presence of B6.K^d APCs (Figure 6-9A), T cells expressing the K^d CAR-eGFP construct proliferated more than the untransduced or K^d ΔCAR-eGFP T cells. However, these differences were very minor and did not reach statistical significance (untransduced versus K^d CAR-eGFP = 1.9-fold increase in proliferation, *p*=0.053; K^d CAR-eGFP versus K^d ΔCAR-eGFP = 1.5-fold increase in proliferation, *p*=0.061), again calling into question the potency of the CAR.

Similar results were obtained when proliferation was analysed in a preliminary experiment by CTV dilution (Figure 6-9B). In this analysis, only proliferation of the $eGFP^+$ cells was assessed, where appropriate. These results demonstrated that the K^d CAR-eGFP T cells proliferated 5.8-fold more than the untransduced T cells and 2-fold more than the K^d ΔCAR-eGFP T cells in the presence of B6.K^d APCs. However, this result was again much more modest than expected as the eGFP gating strategy meant that in theory, 100% of the K^d CAR-eGFP T cells had the potential to be activated in the presence of the B6.K^d APCs. In contrast, TCR-mediated activation through direct allorecognition, achieved by co-culture with BALB/c APCs, would typically be expected to stimulate approximately ~10% of the T cells (Boardman, Jacob et al. 2016). However, dramatically more proliferation (approximately 60%) was observed as a consequence of direct allorecognition than K^d CAR-eGFP engagement (21.1%), suggesting that the CAR did not potently activate the T

cells. Overall, these results suggested that expression of the K^d CAR-eGFP construct did influence the T cells in the presence of K^d but this effect was much more modest than expected, calling into question the potency of this CAR and potentially suggesting that it was not fully functional.

6.3.3 Maintenance and transduction of murine Tregs

6.3.3.1 Characterisation of a previously established Treg line

As discussed above, previous studies performed by the host laboratory have used the Phoenix Eco transduction system to generate B6 Treg lines with indirect allospecificity (Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009) and to generate B6 Tregs which expressed the human sodium-iodide symporter (NIS) for use in an *in vivo* imaging context (Sharif-Paghaleh, Sunassee et al. 2011). In both cases, there was a preference to establish a Treg line by stimulating freshly-isolated $CD4^+CD25^+$ Tregs with DCs for a minimum of 6 consecutive weeks before attempting to transduce these cells. As such, for this thesis, Tregs were used from a cell line previously established by Dr Julia Tsang (MRC Centre for Transplantation, King's College London, UK). This cell line was originally generated by enriching $CD4^+CD25^+$ T cells from the secondary lymphoid organs of B6 mice using a bead-based negative and positive selection protocol. To generate a B6 Treg cell line with self-specificity, these cells were then stimulated with irradiated B6 BM-DCs. The Tregs were re-stimulated once a week and cultured in the presence of 10 U/mL recombinant human IL-2. These cells were then cryopreserved after 6 weeks of expansion.

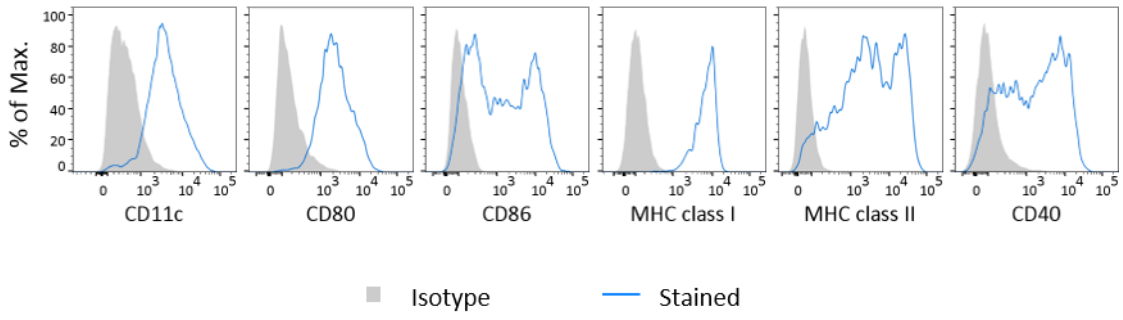

Figure 6-10 | **Phenotype of BM-DCs 6 days post-isolation.** *DC progenitors were enriched from the BM of B6 mice by negative selection, depleting cells which expressed CD4, CD8, B220 and MHC class II. Isolated cells were grown in the presence of GM-CSF for 6 days. The phenotype of these cells was assessed by flow cytometry, using fluorescently-conjugated antibodies which were specific for CD11c, CD80, CD86, MHC class I (K^b), MHC class II ($I\text{-}A^b$) and CD40 (blue lines). Cells stained with isotype controls are shown in solid grey. Data representative of 2 individual experiments.*

Tregs from this previously established self-specific cell line were thawed and stimulated with B6 BM-DCs. These DCs had been enriched as progenitors from the BM of B6 mice and grown in the presence of GM-CSF for 6 days, prior to use. A phenotypic analysis of these BM-DCs was performed by flow cytometry. This demonstrated that the cells expressed the DC marker CD11c as well as I-A^b (MHC class II), CD80 and CD86 which were essential for efficiently activating the $CD4^+$ Tregs (Figure 6-10).

The suppressive capacity of the Treg line was confirmed prior to transduction. This was accomplished by co-culturing the Tregs at a 1:1 ratio with $5x10^4$ $CD4^+$ Tresps in the presence of $1x10^5$ B6 APCs and 1 µg/mL anti-mouse CD3ε (145-2C11 clone) (Golshayan, Jiang et al. 2007, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009). The cells were co-cultured for 72 hours after which Tresp proliferation was measured by ^{3}H-thymidine incorporation. Proliferation of the polyclonally-activated Tresps, measured as cpm, was reduced 23-fold when cultured in the presence of Tregs (1:1 Treg:Tresp ratio; p=0.0023; Figure 6-11). This demonstrated the potent suppressive capacity of these cells *in vitro*.

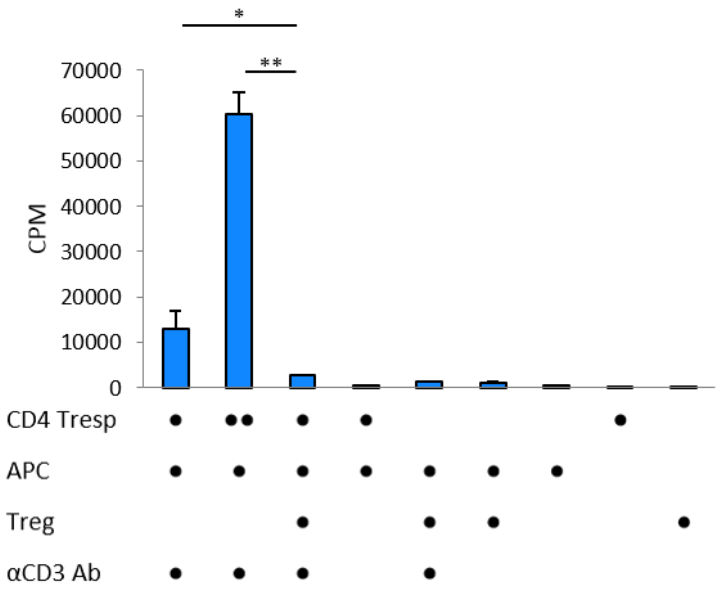

Figure 6-11 | **$CD4^+CD25^+$ Tregs from a previously established cell line efficiently suppressed $CD4^+$ Tresp proliferation.** *Resting $CD4^+CD25^+$ Tregs from a previously established cell line were co-cultured at a 1:1 ratio with $5x10^4$ B6 $CD4^+$ Tresps which were activated by the presence of $1x10^5$ B6 APCs and 1 µg/mL anti-mouse CD3ε (145-2C11 clone). Co-cultures were performed for 72 hours where 1 µCi ^{3}H-thymidine was added for the last 18 hours of culture. Total cell proliferation was measured using beta liquid scintillation counting approach. Significance was determined using a paired two-tailed Student's t-test where *=p<0.05 and **=p<0.01. cpm = counts per minute, ● = $5x10^4$ Tresps, ●● = $1x10^5$ Tresps.*

The Tregs were transduced 2 weeks after they were thawed (8 weeks post-isolation). To achieve this, the Tregs were re-stimulated with irradiated B6-derived BM-DCs and cultured in the presence of 10 U/mL IL-2. These cells were then harvested 48 hours post-stimulation and suspended in fresh Phoenix Eco-derived supernatant containing retroviral particles. The Tregs were maintained in culture for 5 days post-transduction where IL-2 was supplemented every 2 days. The efficiency of transduction was then assessed by flow cytometry, based on eGFP expression. Unfortunately, as the aforementioned studies performed in the host laboratory had previously demonstrated, Tregs from the established cell line were transduced with a low efficiency (0.55 ± 0.48%; n=4; Figure 6-12A). As such, it was necessary to expand these cells for approximately 2 months to obtain a usable number of Tregs. Successfully transduced Tregs were then isolated by cell sorting, based on their eGFP expression, and expanded for an additional 2

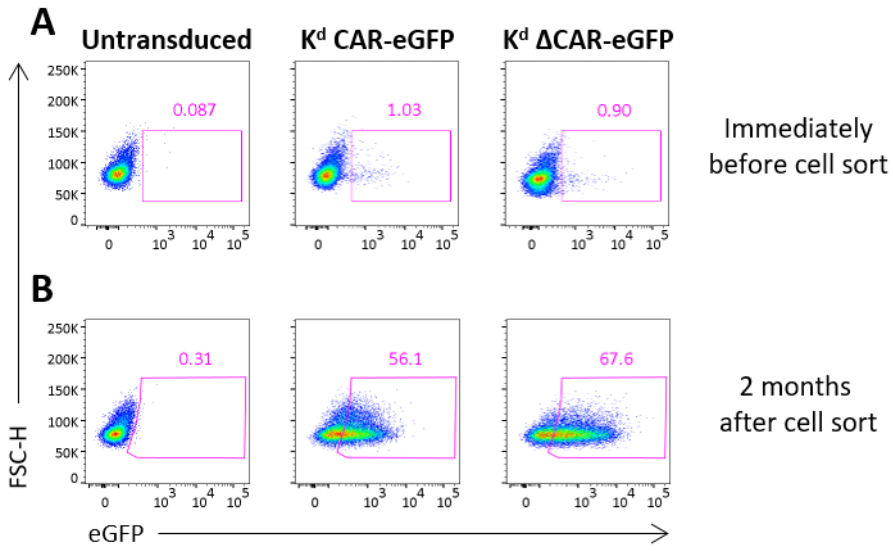

Figure 6-12 | **$CD4^+CD25^+$ Tregs from a previously established cell line were retrovirally transduced to express the K^d CAR-eGFP and K^d ΔCAR-eGFP constructs with a low efficiency.** *A: Transduction efficiency of Tregs from a previously established cell line. $CD4^+CD25^+$ Tregs were stimulated with irradiated B6 BM-DCs and cultured in the presence of 10 U/mL recombinant human IL-2. 48 hours post-stimulation, these Tregs were either left untransduced or retrovirally transduced to express the K^d CAR-eGFP or K^d ΔCAR-eGFP construct. The cells were expanded for an additional 5 days after which the efficiency of transduction was assessed by flow cytometry, based on eGFP expression. B: Transduced cells were expanded for 2 months with repeated stimulations on a weekly basis with irradiated B6 BM-DCs. $eGFP^+$ cells were then enriched by cell sorting. The purity of the end cell product was compromised to ensure a sufficient number of cells was acquired to continue expanding in vitro. These cells were expanded for an additional 2 months after which the proportion of $eGFP^+$ cells was assessed by flow cytometry, as shown. The percentage of events in each gate is provided.*

months to obtain enough cells for downstream applications. Following this 4 month expansion period, the proportion of $eGFP^+$ cells in each Treg line was >50% (Figure 6-12B).

Taken together, these results demonstrated that it was possible to generate a K^d CAR-eGFP Treg line by transducing Tregs from a previously established cell line. However, these cells were transduced with a low efficiency thus multi rounds of expansion were required in order to obtain a practical number of cells for downstream applications.

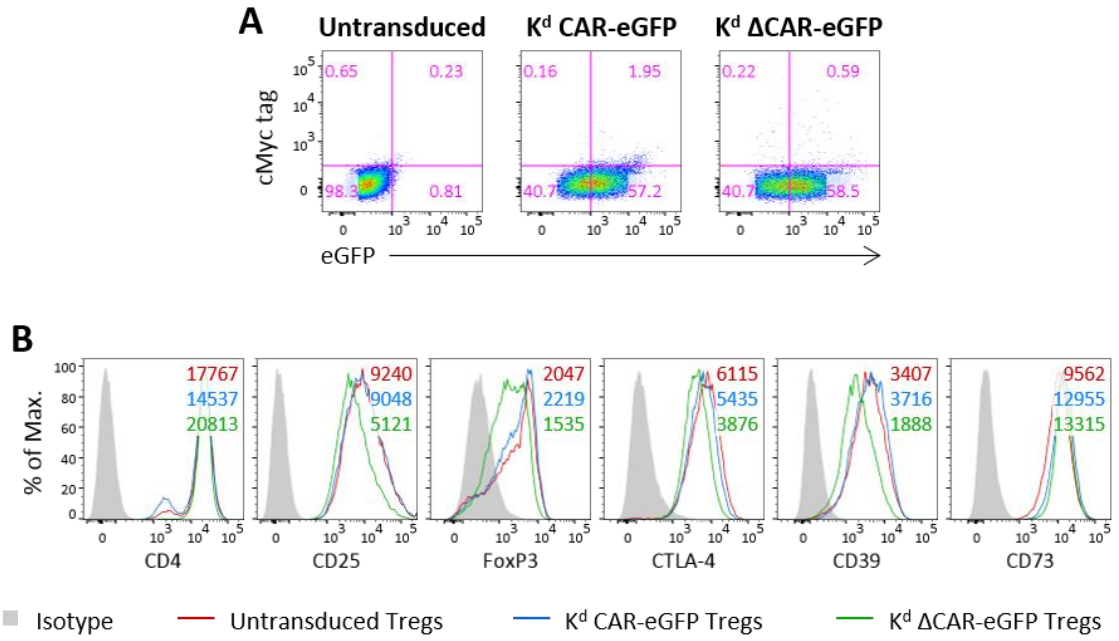

Figure 6-13 | **$CD4^+CD25^+$ Tregs maintained their phenotype following retroviral transduction, cell sorting and a prolonged expansion period.** *A: The presence of the K^d CAR-eGFP and K^d ΔCAR-eGFP proteins on the surface of the sorted Tregs was assessed by flow cytometry using a fluorescently-conjugated antibody specific for the 9E10 cMyc tag epitope present in the extracellular domains of these CARs (Figure 6-3A). The percentage of events in each gate is provided. B: Sorted Tregs were stained with fluorescently-conjugated antibodies specific for the following Treg markers: CD4, CD25, FoxP3, CTLA-4, CD39 and CD73. The expression of these markers by the untransduced (red lines), K^d CAR-eGFP (blue lines) and K^d ΔCAR-eGFP (green lines) Tregs was measured by flow cytometry. MFI values for each of the samples is provided in the top right corner of each plot. Data representative of 2-3 individual experiments.*

6.3.3.2 Retroviral transduction and cell sorting did not influence the phenotype or suppressive capacity of murine $CD4^+CD25^+$ Tregs

As previously mentioned, all of the Tregs lines were expanded for approximately 4 months in order to obtain a sufficient number of K^d CAR-eGFP and K^d ΔCAR-eGFP cells. This prolonged expansion period coupled with the genetic manipulation process had the potential to influence the phenotype or suppressive capacity of these cells. Therefore, in order to confirm that the Tregs maintained their expression of Treg markers and preserved their suppressive capacity, the phenotype and ability of the cells to inhibit $CD4^+$ Tresp proliferation was assessed *in vitro*, as previously described.

Tregs were harvested 7 days post-stimulation and stained with various fluorescently-conjugated antibodies to measure expression of the CAR (cMyc tag) and typical Treg markers. As previously demonstrated with transduced splenocytes (Figure 6-7), there was no detectable cMyc tag on

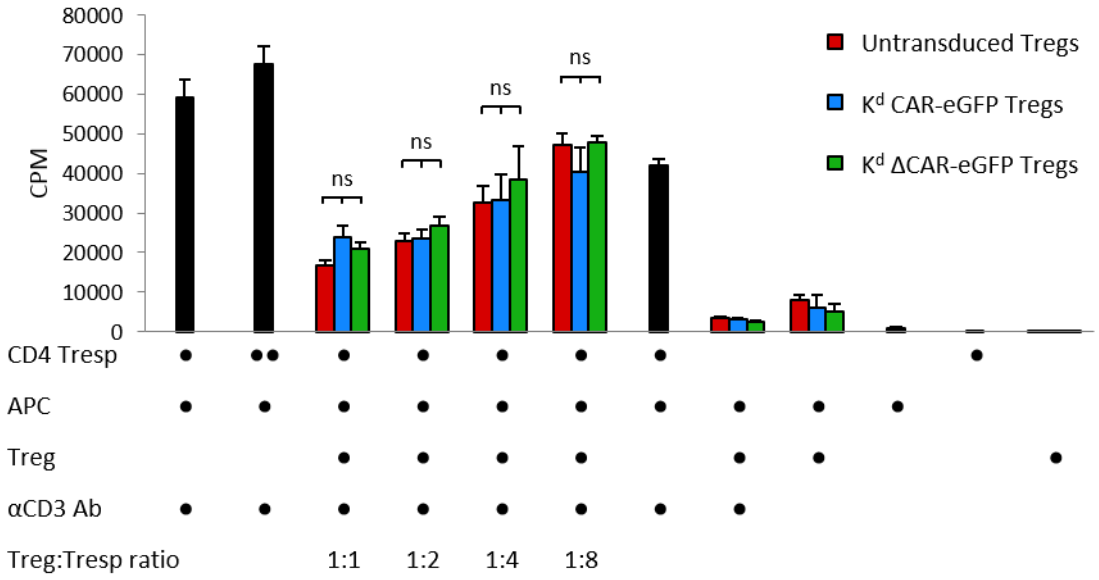

Figure 6-14 | **$CD4^+CD25^+$ Tregs maintained their suppressive capacity in a polyclonal manner following retroviral transduction, cell sorting and a prolonged expansion period.** *$5x10^4$ B6 $CD4^+$ Tresps were stimulated with $1x10^5$ B6 APCs and 1 µg/mL anti-mouse CD3ε (145-2C11 clone). Resting $CD4^+CD25^+$ Tregs which were untransduced (red bars), or engineered to express the K^d CAR-eGFP (blue bars) or K^d ΔCAR-eGFP (green bars) construct were added to the co-culture at various ratios. Co-cultures were performed for 72 hours where 1 µCi 3H-thymidine was added for the last 18 hours of culture. Total cell proliferation was measured using beta liquid scintillation counting approach. A two-tailed paired Student's t-test was performed but no significant differences were observed. Data representative of 2 individual experiments. cpm = counts per minute, ns = not significant, ● = $5x10^4$ Tresps, ●● = $1x10^5$ cells.*

the surface of the $eGFP^+$ Tregs when assessed using a fluorescently-conjugated cMyc tag-specific antibody (Figure 6-13A). Fortunately, the transduction and sorting processes were found to have not influenced the phenotype of the Tregs as the untransduced and K^d CAR-eGFP Tregs expressed similar levels of CD4, CD25, FoxP3, CTLA-4, CD39 and CD73 (Figure 6-13B). However, the K^d ΔCAR-eGFP Tregs did express lower levels various markers compared to the other two Treg lines including CD25 (MFI = 5,121 compared to >9,000), FoxP3 (MFI = 1,535 compared to >2,000) and CD39 (1,888 compared to >3,400), suggesting that the prolonged expansion period may have begun to influence the phenotype of this cell line.

To compare the suppressive capacity of the 3 Treg lines, a polyclonal suppression assay was performed where the Tregs were co-cultured with Tresps at various ratios with Tresps and B6 APCs in the presence of 1 μg/mL anti-mouse CD3ε, as previously described. Proliferation was measured after 72 hours of culture by ^{3}H-thymidine incorporation. Overall, the transduced Tregs exhibited a similar suppressive capacity to the untransduced Tregs, demonstrating that the transduction and subsequent cell sorting processes had not influenced the functionality of these cells (Figure 6-14). However, relative to the suppressive capacity these cells exhibited prior to the transduction and expansion processes (Figure 6-11), each line appeared to be less potent. Previously, at a 1:1 Treg:Tresp ratio, the Tregs inhibited Tresp proliferation (measured as cpm) by 23-fold prior to the expansion. However, the same (untransduced) Tregs co-cultured at the same ratio only inhibited Tresp proliferation by 4.1-fold following expansion.

Taken together, these results demonstrated that retroviral transduction of murine $CD4^+CD25^+$ Tregs did not influence the phenotype or suppressive capacity of these cells. The subsequent prolonged expansion period which was necessary to acquire a practical number of cells may have influenced the functionality of these cells, independently of the transduction process.

6.3.4 Functional assessment of the K^d-specific CAR expressed by Tregs

6.3.4.1 K^d CAR-eGFP Tregs preferentially proliferated in the presence of the K^d target antigen

In a previous study published by the host laboratory, Tregs with indirect allospecificity were shown to proliferate *in vivo* when injected into transgenic B6.K^d mice but not in B6 mice (Tsang, Tanriver et al. 2008). To investigate whether K^d CAR-eGFP Tregs responded in a similar manner, B6 and B6.K^d mice were injected i.v. with $2x10^6$ CTV-labelled Tregs. The mice were sacrificed after

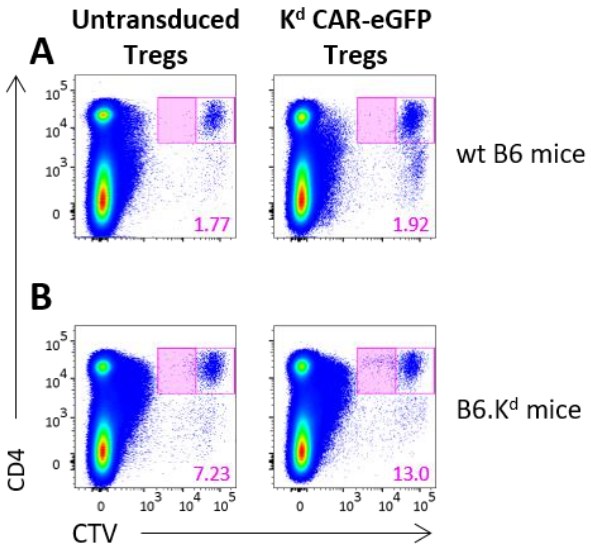

Figure 6-15 | **K^d CAR-eGFP Tregs preferentially proliferate upon transfer into B6.K^d mice, compared to B6 mice.** *$2x10^6$ untransduced or Kd CAR-eGFP Tregs were adoptively transferred into wt B6 or B6.K^d mice by iv injection. Mice were scarified after 72 hours and spleens were harvested to measure Treg proliferation. Total splenocytes were stained with a fluorescently-conjugated anti-mouse CD4 and Treg proliferation was assessed using a flow cytometer. Transferred Tregs were identified by their expression of CD4 and CTV label (gate shown). % proliferation of these cells was calculated as the proportion of divided Tregs (shaded region of the gate) relative to the total number of transferred cells (entire gate). % proliferation values are provided in the bottom right corner of each plot. Data shown is representative of 2 mice per group in a preliminary experiment which was performed once. Total data is summarised in Table 6-1.*

72 hours and the proliferation of the transferred Tregs present in the spleens was assessed by flow cytometry, as determined by CTV dilution.

Transferred Tregs were identified in the total splenocytes by their expression of CD4 and CTV label. A minimal level of proliferation was recorded for both the untransduced and K^d CAR-eGFP Tregs when injected into B6 mice (Figure 6-15A and Table 6-1). In contrast, a noticeable level of proliferation was observed when these cells were transferred into B6.K^d mice (Figure 6-15B and Table 6-1). Untransduced Tregs proliferated 4-fold more in the B6.K^d mice compared to B6 mice, demonstrating a high level of background proliferation. K^d CAR-eGFP Tregs improved on this difference, proliferating 8.5-fold more in the B6.K^d mice compared to the B6 mice suggesting that expression of the K^d CAR-eGFP construct did confer an advantage. However, given the high level of background proliferation in this assay and very minor increase in K^d CAR-eGFP Treg proliferation that was observed in the B6.K^d mice, relative to this background, it was clear that the K^d CAR-eGFP construct was not having the desired potent effect one would have expected. Overall, these results suggested that Tregs expressing the K^d CAR-eGFP construct preferentially

Recipient Mouse	Treg line injected	% Treg proliferation		
		Mouse 1	Mouse 2	Average
wt B6	Untransduced	1.77	N/A	1.77
	K^d CAR-eGFP	1.56	1.92	1.74
B6.K^d	Untransduced	7.99	7.23	7.61
	K^d CAR-eGFP	16.5	13.0	14.75

Table 6-1 | **Summary of the % of Treg proliferation upon adoptive transfer into B6 and B6.K^d mice (Figure 6-15).**

expanded in the presence of K^d *in vivo*, but this was not achieved at an extent which unequivocally demonstrated the functionality of the K^d CAR-eGFP construct.

6.3.4.2 K^d CAR-eGFP Tregs were not more suppressive than polyclonal Tregs in the presence of K^d-expressing APCs

As K^d CAR-eGFP Tregs preferentially proliferated in the presence of K^d, this suggested that the CAR was functional. To confirm this, a linked antigen-specific suppression assay was performed. The ability of untransduced, K^d CAR-eGFP and K^d ΔCAR-eGFP Tregs to suppress antigen-specific $CD4^+$ Tresp proliferation in the presence of K^d was compared. In this assay, $CD4^+$ Tresps were isolated from transgenic OT-II mice, a B6 mouse strain which expresses a transgenic TCR that is specific for the chicken ovalbumin peptide ($OVA_{323-339}$) presented in the context of I-A^b. OT-II Tresps were stimulated with B6.K^d APCs which were pulsed with 0.2 µg/mL $OVA_{323-339}$ peptide. Tregs were then added to the co-culture at various Treg:Tresp ratios to assess whether the K^d CAR-eGFP Tregs inhibited OVA-specific Tresp proliferation more effectively than the untransduced Tregs in the presence of K^d.

When Tresp proliferation was measured by ^{3}H-thymidine incorporation, all three of the Treg lines exhibited a minimal level of suppression (Figure 6-16). However, a high cpm count was observed in wells that did not contain Tresps, suggesting that the suppressive ability of the Tregs was being masked by their own proliferation. This was confirmed when the proliferation of the Tresps was measured by CTV dilution (Figure 6-17). In this setting, all of the Tregs were shown to efficiently inhibit Tresp proliferation, affirming their suppressive capacity. However, no statistically significant differences were observed in the level of suppression exhibited by the three Treg lines,

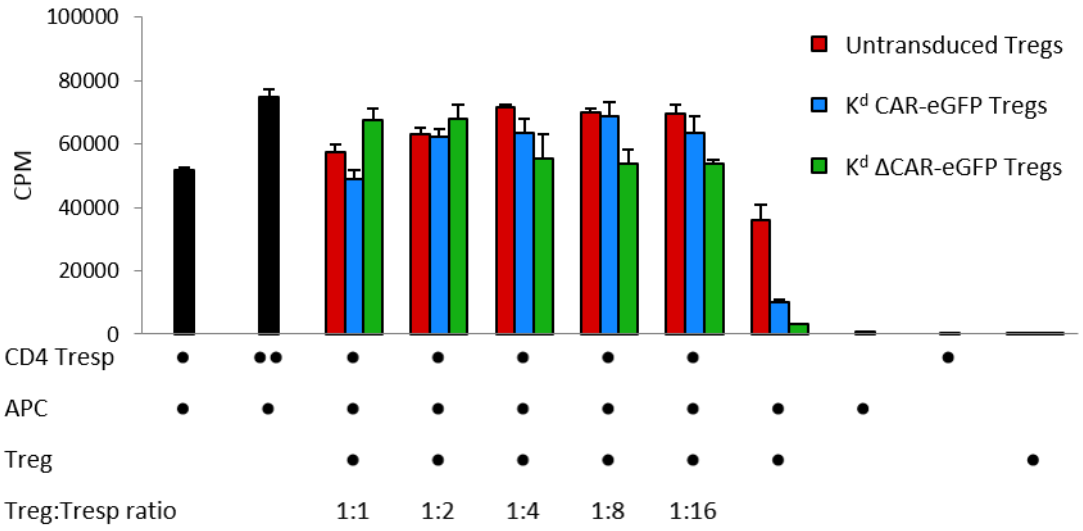

Figure 6-16 | **K^d CAR-eGFP Tregs did not suppress $CD4^+$ Tresp proliferation more effectively than untransduced Tregs in the presence of K^d-expressing APCs.** *A linked suppression assay was performed where $5x10^4$ $CD4^+$ Tresps from OT-II mice were co-cultured with $1x10^5$ B6.K^d APCs pulsed with 0.2 µg/mL $OVA_{323-339}$. Untransduced (red bars), K^d CAR-eGFP (blue bars) and K^d ΔCAR-eGFP (green bars) Tregs were added to the co-culture at various Treg:Tresp ratios. Co-cultures were performed for 72 hours where 1 µCi ^{3}H-thymidine was added for the last 18 hours of culture. Total cell proliferation was measured using a beta liquid scintillation approach. Data represents mean + SD of a single preliminary experiment. cpm = counts per minute.*

at any of the Treg:Tresp ratios analysed. At a 1:4 Treg:Tresp ratio, the % suppression exhibited by the untransduced, K^d CAR-eGFP and K^d ΔCAR-eGFP Tregs was 38.3 ± 3.1%, 30.3 ± 1.0% and 32.1 ± 6.9%, respectively. Taken together with the overall suppressive profile of the three Treg lines, these results suggested that the K^d CAR-eGFP Tregs did not possess a superior suppressive capacity over untransduced Tregs in the presence of K^d. In this assay, the TCR-dependent self-specificity of the Tregs likely contributed to "background suppression". However, it was expected that expression of the K^d CAR-eGFP construct would contribute in a cumulative manner to this self-specificity, providing the K^d CAR-eGFP Tregs with an added advantage, but this was not observed. Overall, these results demonstrated that in the presence of B6.K^d APCs, Tregs expressing the K^d CAR-eGFP were unable to suppress $CD4^+$ Tresp proliferation more effectively than untransduced Tregs.

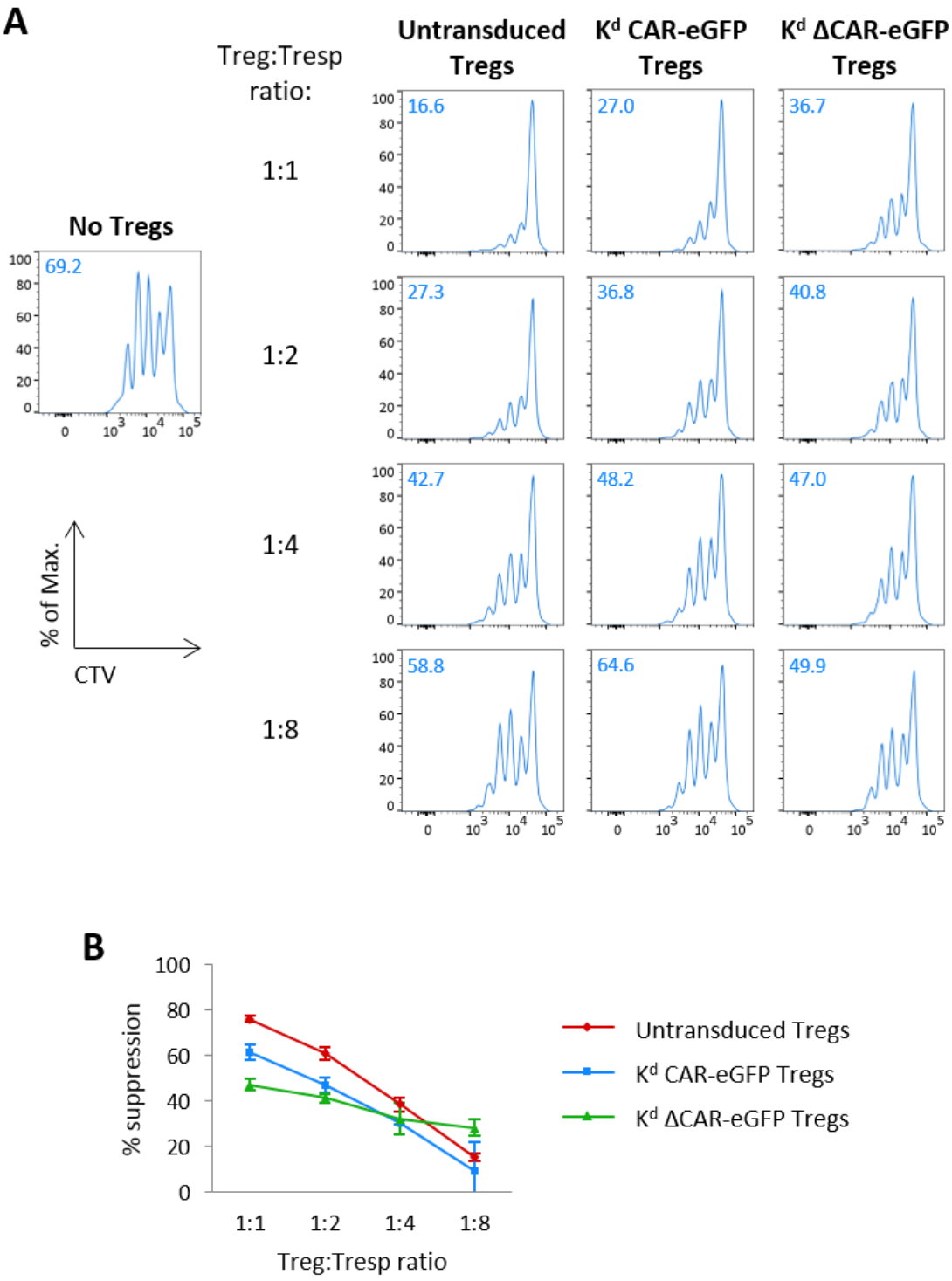

Figure 6-17 | **K^d CAR-eGFP Tregs did not suppress $CD4^+$ Tresp proliferation more effectively than untransduced Tregs in the presence of K^d-expressing APCs.** *A linked suppression assay was performed where $5x10^4$ CTV-labelled $CD4^+$ Tresps from OT-II mice were co-cultured with $1x10^5$ B6.K^d APCs pulsed with 0.2 µg/mL $OVA_{323-339}$. Untransduced (red), K^d CAR-eGFP (blue) and K^d ΔCAR-eGFP (green) Tregs were added to the co-culture at various Treg:Tresp ratios. Co-cultures were performed for 72 hours and Tresp proliferation was measured using CTV dilution by flow cytometry (A). B: Data from technical replicates was pooled. % Tresp proliferation was calculated using division indices, relative to Tresps cultured alone. % suppression was then calculated as the inverse of % Tresp proliferation. Data represents mean ± SD of a single preliminary experiment. No statistical differences were observed at any Treg:Tresp ratio analysed (paired two-tailed Student's t-test).*

6.4 DISCUSSION

In this chapter, a novel second generation CAR specific for K^d was constructed with the aim of generating a mouse MHC class I-allospecific Treg cell line. The rationale behind this was to compare the efficacy with which CAR Tregs and Tregs with alternative allospecificities protected against graft rejection. The antigen-targeting moiety of this CAR incorporated the V_H and V_L chain sequences of a well characterised K^d-specific antibody clone (SF1-1.1.10). This scFv sequence was linked to an intracellular murine CD28-CD3ζ signalling domain with a C-terminal eGFP fusion reporter gene (K^d CAR-eGFP). A second truncated K^d-specific CAR which lacked the capacity to signal intracellularly was generated by removing the CD28-CD3ζ coding sequence from the original CAR ORF (K^d ΔCAR-eGFP).

Total splenocytes were efficiently transduced to express both of these constructs (29.4 ± 17.2%). Upon co-culture with B6.K^d APCs, K^d CAR-eGFP T cells proliferated more than untransduced or K^d ΔCAR-eGFP T cells, suggesting that the K^d CAR-eGFP was functional but given the minor difference which was observed, it was evident that the K^d CAR-eGFP construct was not potent. $CD4^+CD25^+$ Tregs from a previously established cell line were then transduced to express the K^d CAR constructs with a low efficiency (0.55 ± 0.48%). This process did not influence the phenotype or suppressive capacity of the Tregs. K^d CAR-eGFP Tregs were shown to preferentially proliferate in the presence of K^d when adoptively transferred into B6.K^d mice, although the increase in proliferation achieved was minimal compared to the high levels of background proliferation which were also observed. Unfortunately, proceeding results demonstrated that K^d CAR-eGFP Tregs did not inhibit $CD4^+CD25^-$ Tresp proliferation more effectively than untransduced self-specific or K^d ΔCAR-eGFP Tregs in the presence of B6.K^d APCs. These findings demonstrated that expression of the K^d CAR-eGFP construct did not confer a biologically-relevant specificity for K^d and suggested that this CAR possessed a very low potency.

One issue which was not categorically resolved in this chapter was the fact that cMyc tag staining was not detectable on transduced K^d CAR-eGFP-expressing T cells or Tregs. The concept of exploiting a cMyc tag to detect CAR expression was successfully demonstrated in Chapters III and IV and has previously been shown by Maher *et al*. (unpublished data) and MacDonald *et al*. (MacDonald, Hoeppli et al. 2016). However, despite the 9E10 epitope being incorporated into the K^d CAR-eGFP construct, cMyc tag staining was not detectable in cells which expressed this construct. This suggested that either the K^d CAR-eGFP construct was not trafficked to the cell surface or that steric hindrance of the scFv was blocking the 9E10 epitope. Experiments using confocal microscopy suggested that the K^d CAR-eGFP construct was trafficked to the cell membrane, thus blocking of the 9E10 epitope was to blame for a lack of cMyc tag detection.

However, this could have more simply been resolved by staining transduced cells with a K^d-based tetramer. Positive staining would have confirmed that the CAR was present on the cell surface and that it facilitated the recognition of K^d. However, this approach was not possible due to a lack of a K^d-based tetramer.

Many of the assays described in this chapter were plagued by a high level of background responses. The first approach which was employed to assess the functionality of the K^d CAR-eGFP construct was to investigate whether K^d CAR-eGFP T cells preferentially proliferated in the presence of B6.K^d APCs, compared to B6 APCs. However, high levels of background proliferation were observed in these experiments, particularly when control T cells were co-cultured with B6.K^d and BALB/c APCs. This could be attributed to TCR-mediated stimulation of approximately 10% of the T cells which possessed direct allospecificity (Boardman, Jacob et al. 2016). To circumvent this issue, a similar experimental setup could be applied to investigate the proliferation of $CD4^+$ T cells which are co-cultured with MHC class II-deficient K^d-expressing APCs. The depletion of $CD8^+$ T cells would prevent background proliferation of cells stimulated by the K^d molecule whilst the absence of MHC class II would limit TCR-mediated stimulation of the remaining $CD4^+$ T cells. Experiments using $CD4^+CD25^-$ T cells were performed but unfortunately, no differences were observed in the proliferation induced by stimulation with B6 or B6.K^d APCs. Taken together with the subsequent results which implied that the K^d CAR-eGFP construct was functional, this suggested that to obtain optimal results in this setup, MHC class II-deficient K^d-expressing APCs were required. However, MHC class II-deficient mice were unfortunately not available.

Experiments assessing the suppressive efficacy of the K^d CAR-eGFP Tregs in the presence of K^d-expressing APCs were also complicated by a high level of background responses. In this setup, $CD4^+$ Tresps from OT-II mice were stimulated with OVA peptide-pulsed B6.K^d APCs. B6 Tregs which were either untransduced or modified to express a K^d-specific CAR were co-cultured the Tresps and APCs to investigate the efficacy with which these cells inhibited Tresp proliferation. However, these Tregs were likely to express TCRs with a high specificity for self-I-A^b, due to being stimulated with B6 DCs. As such, the K^d CAR-eGFP Tregs could have been activated by the B6.K^d APCs in a CAR-independent manner. Tsang *et al*. resolved this issue by using $CD4^+$ Tresps and APCs from alternative transgenic mice (Tsang, Tanriver et al. 2008). DO11.10 mice are BALB/c mice which are transgenic for the same OVA-specific TCR genes which are possessed by OT-II mice (Robertson, Jensen et al. 2000). Tsang *et al*. performed a suppression assay in which DO11.10 $CD4^+$ Tresps were stimulated with $OVA_{323-339}$ peptide-pulsed BALB/c APCs. Consequently, in the absence of B6 I-A^b-expressing APCs, only Tregs with direct allospecificity were stimulated through their TCR, limiting the background suppression which was observed. This approach was not possible due to the lack of DO11.10 mice available but is a fundamental experiment which should

be performed in the future to assess whether expression of the K^d CAR-eGFP does indeed confer a functional advantage in the presence of K^d.

An additional concern raised from the experiments performed in this chapter surrounded the efficacy of the K^d CAR-eGFP construct. Results from the aforementioned T cell proliferation assays suggested that the K^d CAR-eGFP construct may have been functional, but the low level of proliferation which was observed in these experiments called into question the efficacy of the CAR. This was particularly true given the fact that significantly more proliferation was induced through direct allorecognition in <10% of the cells (co-culture with BALB/c APCs) than through the CAR in ~30% of the cells (co-culture with B6.K^d APCs).

In 2010, Kochenderfer *et al*. generated and published the sequence of a murine CD19-specific CAR which incorporated a CD28-CD3ζ signalling domain (Kochenderfer, Yu et al. 2010). A comparison of this sequence to the K^d CAR-eGFP sequence demonstrated that the hinge, transmembrane and signalling domains of these CARs were 99% identical. This suggested that the low efficacy of the K^d CAR-eGFP could be attributed either to a sub-optimal antigen recognition domain or the two amino acid residues which differed between the CD19-specific CAR and the K^d CAR-eGFP construct. Both of these options are discussed below.

The simplest explanation to account for the low efficacy of the K^d CAR-eGFP construct was that the CAR did not efficiently bind K^d. The length of the hinge domain can influence this process, particularly when a target epitope is close to the cell membrane (discussed in section 1.3.1.2) (Guest, Hawkins et al. 2005). Consequently, when targeting an MHC class I molecule, the ideal MHC subunits to target are the α1 and α2 subunits as these are the subunits which are most exposed and accessible. Indeed, the A2 CARs constructed in Chapter III and by MacDonald *et al*. both targeted different epitopes of the HLA-A2 α2 subunit (shown in Figure 3-21B). However, the SF1-1.1.10 scFv clone used to generate the K^d CAR-eGFP construct recognised an epitope in the α3 subunit of the K^d molecule (Abastado, Ojcius et al. 1993, Noun, Reboul et al. 1996, Noun, Reboul et al. 1998). Consequently, it is a distinct possibility that the hinge domain incorporated into the K^d CAR-eGFP construct was not of a sufficient length to allow for optimal engagement of the SF1-1.1.10 epitope in the K^d molecule. This is a fundamental point to address in the future which cannot be resolved through tetramer staining.

An alternative explanation for the low efficacy of the K^d CAR-eGFP is that the scFv domain itself did not efficiently engage K^d. Indeed, this antigen recognition domain was designed and generated *de novo* using V_H and V_L chain sequences which were obtained from a K^d-specific antibody. It is well established that the tertiary structure adopted by these chains can differ when they are incorporated into an scFv or a complete antibody (Argos 1990, Smallshaw, Georges et

al. 1999). As such, a loss of specificity can be potentiated when these sequences are linked in an scFv format. Furthermore, previous studies have demonstrated a clear link between the affinity with which a CAR engages its target antigen and the response elicited (Liu, Jiang et al. 2015).

A third explanation for the low efficacy of the K^d CAR-eGFP construct related to work which was published by Nguyen *et al*. in 2003 (Nguyen, Moisini et al. 2003). This study set out to investigate whether CARs could be used to specifically target and destroy $CD8^+$ T cells with direct allospecificity in a transplant context. To achieve this, a murine CAR was constructed which presented elements of a K^b complex. Specifically, this CAR incorporated the extracellular and transmembrane domains of the K^b molecule (Geiger, Nguyen et al. 2001) which were linked to an intracellular CD28-CD3ζ signalling domain. The rationale behind this was that in a transplant setting, CAR-expressing T cells would specifically engage and destroy $CD8^+$ T cells with direct allospecificity. In this study, CAR T cells which were pulsed with $OVA_{257-264}$ peptide were shown to specifically kill OT-I T cells (TCR specific for K^b-OVA) *in vitro* and *in vivo*. However, these findings were shadowed by the discovery of a noncanonical dileucine internalisation motif which was present in the CD28 portion of their CAR. These authors demonstrated that mutating this LL motif into a GG motif increased detection of their CAR on the cell surface by up to 4-fold. When designing the K^d CAR-eGFP construct, this LL motif was not mutated and as such, was incorporated into the end product. Rapid internalisation of the K^d CAR-eGFP protein would account for both the limited efficacy of this CAR and the lack of cMyc tag staining which was discussed above. The effects of this motif can be determined in the future by generating a K^d CAR-eGFP construct with point mutations in this LL motif.

6.4.1 Summary

The work presented in this chapter aimed to generate a murine K^d-specific CAR with the rationale of comparing the efficacy with which MHC class I-allospecific CAR Tregs and Tregs with alternative allospecificies inhibited transplant rejection. A CAR was generated for this purpose but the functionality of this CAR in the presence of K^d-expressing cells was unconvincing. K^d CAR-eGFP Tregs proliferated marginally more in the presence of K^d but no definitive functional advantage was exhibited by the expression of this molecule.

CHAPTER VII

GENERAL DISCUSSION AND FUTURE WORK

7.1 GENERAL DISCUSSION

The safety and efficacy of Treg therapy is currently being assessed clinically as a means of promoting transplant tolerance in kidney and liver transplant recipients (Table 1-1). For this purpose, the successful generation and expansion of graft-specific Tregs is desirable as these cells promote transplant tolerance more effectively than polyclonal Tregs (Sagoo, Lombardi et al. 2008, Tsang, Tanriver et al. 2008, Tsang, Tanriver et al. 2009, Putnam, Safinia et al. 2013). Allospecific Tregs can be generated using GMP-compatible protocols by stimulating Tregs with allogeneic APCs (Peters, Hilbrands et al. 2008, Tu, Lau et al. 2008, Chen, Delgado et al. 2009, Zheng, Liu et al. 2010, Sagoo, Ali et al. 2011, Noyan, Lee et al. 2013, Putnam, Safinia et al. 2013, Cherai, Hamel et al. 2015, Jeffery, Braitch et al. 2016). However, the Tregs resulting from this process are restricted to recognising allogeneic peptides presented in the context of allo-MHC class II molecules. This thesis explored a novel approach which may be employed to confer peptide-independent donor MHC class I-allospecificity onto Tregs using CAR technology.

Novel CARs were used to direct the specificity of human and mouse Tregs towards the allogeneic MHC class I molecules HLA-A2 and K^d, respectively. For the human Tregs, CAR delivery was achieved through lentiviral transduction which was shown to not influence the phenotype or suppressive ability of these cells *in vitro*. A2 CAR-eGFP Tregs were shown to suppress autologous $CD4^+CD25^-$ Tresp proliferation more effectively than polyclonal or A2 ΔCAR-eGFP Tregs in the presence of HLA-$A2^+$ APCs, without eliciting cytotoxicity. Furthermore, Tregs expressing a HLA-A2-specific CAR were found to favour transmigration into HLA-$A2^+$ tissues in manner which was independent of CAR signalling and Treg activation. This observation was supplemented by the finding that these cells were also retained in HLA-$A2^+$ skin allografts *in vivo*. Most importantly, A2 CAR-eGFP Tregs were shown to protect human HLA-$A2^+$ skin grafts more effectively than polyclonal Tregs, making the argument for the adaptation of CAR Tregs in the clinic.

Work with the murine K^d CAR-eGFP Tregs is still ongoing but results thus far have demonstrated that this CAR can be delivered into murine Tregs without influencing the phenotype or suppressive capacity of these cells. Unfractioned T cells expressing the K^d CAR-eGFP construct appeared to proliferate more in the presence of B6.K^d APCs than B6 APCs *in vitro* although this increase in proliferation was minimal and difficult to decipher over the high level of background proliferation which was observed in these assays. Similarly, K^d CAR-eGFP Tregs may have proliferated more when adoptively transferred into B6.K^d mice, compared to B6 mice, although this increase was also minor and obscured by a high level of background proliferation. However, the superior suppressive capacity of K^d CAR-eGFP Tregs compared to self-specific Tregs in the presence of B6.K^d APCs remained to be demonstrated.

The concept of redirecting Tregs towards MHC class I molecules has previously been explored using TCRs. Brusko *et al*. and Plesa *et al*. both demonstrated that functional HLA-A2-restricted $CD4^+$ Tregs could be generated by lentivirally transducing Tregs to express specific $CD8^+$ T cell-derived TCR α and β chain genes (Brusko, Koya et al. 2010, Plesa, Zheng et al. 2012). These studies demonstrated that it was possible to lentivirally transduce human Tregs to confer antigen-specificity without influencing the phenotype or suppressive capacity of these cells, as was confirmed in Chapter IV. Tregs expressing these MHC class I-specific TCRs were shown to efficiently suppress the activation and proliferation of Tconvs with the same specificity both *in vitro* and *in vivo*. More importantly however, these studies highlighted the concept of redirecting Tregs towards MHC class I antigens which are prevalently presented in target tissues, as opposed to the MHC class II antigens which are typically targeted by Tregs. However, this approach was compromised as only specific TCRs which bound the target MHCs with a very high affinity could be used, due to the lack of CD8 stabilisation in the TCR:MHC interaction (Plesa, Zheng et al. 2012). Furthermore, these cells were still restricted to recognising peptides presented in the context of MHC molecules. In contrast, CARs can incorporate antibody-derived antigen recognition domains which engage donor MHC molecules in a peptide-independent manner. Consequently, conferring specificity through the use of CAR technology is the more prudent choice for generating graft-specific Tregs as these cells do not rely on the presentation of specific peptides in order to engage their target antigen, unlike TCR-manipulated Tregs.

A widely reported limitation of CAR technology is the fact that these proteins are restricted to recognising extracellular target antigens (Morris and Stauss 2016). In contrast, TCRs can recognise peptides derived from intracellular antigens. This is a particular limitation in cancer research where tumour associated antigens (TAA) may not be presented extracellularly, allowing cancerous cells to evade immune recognition. Conversely, in a transplantation context, donor MHC class I molecules are ubiquitously expressed in allografts as well as on donor-derived APCs and cross-dressed APCs (section 1.1.1.3.1). As such, the availability of the target antigen is not an issue. However, this consistent antigen availability may be a double-edged sword as Tregs are indeed activated within the graft but consistent activation may potentially lead to activation-induced cell death (AICD). In cancer research, T cells expressing hyperactive CARs have been shown to be highly susceptible to AICD as a consequence of high FasL expression (Lanitis, Poussin et al. 2013, Kunkele, Johnson et al. 2015). Although it has been suggested that Tregs are less susceptible to AICD than Tconvs (Yolcu, Ash et al. 2008), this remains a caveat of CAR technology which warrants further investigation in Tregs.

The data presented in Chapter V suggested that AICD of CAR Tregs was not a pressing issue and that these cells indeed survived long-term in the investigated setting. HLA-$A2^+$ skin grafts of mice

which received PBMCs with A2 CAR-eGFP Tregs contained more $CD3^+FOXP3^+$ cells than the grafts of mice which received PBMCs with polyclonal Tregs, suggesting that the transferred Tregs survived long-term (more than 6 weeks; Figure 5-14). However, a future analysis to measure the presence of the cMyc tag or eGFP is required to confirm this. The long-term Treg survival achieved was contrary to the results of MacDonald *et al*. who investigated the capacity of A2 CAR Tregs to inhibit xeno-GvHD (MacDonald, Hoeppli et al. 2016). In their setting, A2 CAR Tregs were not detected in circulation 2 weeks following adoptive transfer which was attributed to a lack of IL-2 in the *in vivo* system. Indeed, this dependence on IL-2 for Treg survival is currently being addressed clinically where trials are investigating the use of low-dose IL-2 therapy to promote *in vivo* Treg expansion for treating transplant rejection and autoimmunity (Koreth, Matsuoka et al. 2011, Saadoun, Rosenzwajg et al. 2011, von Spee-Mayer, Siegert et al. 2016). MacDonald *et al*. proceeded to demonstrate that STAT5, a downstream target of IL-2, was not phosphorylated in response to CAR stimulation (MacDonald, Hoeppli et al. 2016). STAT5 phosphorylation is fundamental for the function and survival of Tregs (Passerini, Allan et al. 2008, Wuest, Willette-Brown et al. 2008, Vogtenhuber, Bucher et al. 2010, Bailey-Bucktrout, Martinez-Llordella et al. 2013, Mahmud, Manlove et al. 2013). However, this was not an issue in the experiments performed in Chapter V. This difference between systems may be explained by the possibility that in the xeno-GvHD setting, protection may have been mediated through the inhibition of Tconv engraftment thus these cells were not available to produce IL-2. In contrast, in the experiments performed in chapter V, the presence of the allogeneic skin graft facilitated the activation of Tconvs which produced IL-2, aiding Treg survival. Treg dependence on IL-2 could be circumvented by co-transducing these cells to express a chimeric cytokine receptor which delivers intracellular IL-2R signals upon recognition of a prevalent antigen or alternative cytokine (Wilkie, Burbridge et al. 2010, Leen, Sukumaran et al. 2014).

Another mechanism which has been shown to reduce the longevity of CAR-expressing T cells is xenogeneic recognition of the exposed antigen targeting moiety of the CAR. This issue has previously undermined two clinical trials in which transferred T cells expressing a CAR that contained a murine-derived scFv did not persist long-term post-transfer (Kershaw, Westwood et al. 2006, Lamers, Willemsen et al. 2011). This was attributed to the *in vivo* generation of antibodies which specifically recognised the CARs. The A2 CAR-eGFP construct designed in Chapter III incorporated an scFv which was derived from human origins (Watkins, Brown et al. 2000). This reduced the immunogenicity of the CAR and improved the chances of the CAR Tregs surviving long-term post-transfer. However, it is imperative to note that the immunogenicity of this CAR was not addressed in the experiments performed in Chapter V and in humans, the generation of anti-A2 CAR antibodies remains a potential problem. Indeed, CAR Tregs were

shown to survive long-term in the BRG mice which were injected with human PBMCs but a severe limitation of this system is that B cells do not engraft thus it was not possible for anti-A2 CAR antibodies to be generated (Ali, Flutter et al. 2012). This lack of B cells, or any other APCs for that matter, also had important implications for investigating the responses of T cells and Tregs with indirect allospecificity as these cells are unable to be stimulated in these mice. Consequently, a different model would be required to compare the protective efficacy of Tregs with different allospecificities. The anticipated mouse-based experiments described in Chapter VI had the potential to address these issues but were unfortunately not performed due to complications in generating a potent K^d-specific CAR.

7.2 FUTURE WORK

In this thesis, the expression of a donor MHC class I-specific CAR was shown to confer a functional advantage to polyclonal Tregs in a transplant context. However, there are still some major obstacles which must be addressed before this approach is adopted in the clinic. The most pressing of these is whether this approach is the most prudent choice for generating graft-specific Tregs. Indeed, A2 CAR-eGFP Tregs were shown to inhibit alloimmune-mediated injury of HLA-$A2^+$ skin grafts more effectively than polyclonal Tregs, but it remains unclear how these cells compare to Tregs with direct or indirect allospecificity. The work of Chapter VI set out to address this but unfortunately, ambiguity in the functionality of the K^d CAR-eGFP construct meant that this was not achieved.

The safety and efficacy of Tregs with direct allospecificity at reducing kidney and liver transplant rejection is currently being assessed outside of the UK (Table 1-1). In contrast, evidence for the efficacy of CAR Tregs is only just emerging in pre-clinical studies. As such, it is clear that pre-clinical studies must first compare the efficacy of these cells before the idea of CAR Tregs is entertained in transplantation-based clinical trials. Furthermore, CAR Tregs must be shown to protect allografts dramatically more effectively than Tregs with direct allospecificity, as CAR Tregs are associated with additional genetic manipulation-based risks. To this end, the efficacy of CAR Tregs may be improved by employing approaches which are currently being investigated in cancer research. As discussed in section 1.3.3, TRUCK and Armoured CAR T cells have been developed to express additional genes which are delivered concurrently with the CAR (Chmielewski, Hombach et al. 2014, Yeku and Brentjens 2016). The concept of the inducible IL-12 cassette is of particular interest as this facilitated CAR T cells to secrete high levels of IL-12 following CAR engagement. This idea could be adapted to CAR Tregs such that they secrete high levels of IL-10 or alternative

anti-inflammatory cytokines when activated. Additional manipulations to enhance the stability and/or functionality of these cells may also be considered. This could be achieved by co-transducing the Tregs to express exogenous *FOXP3* (Fransson, Piras et al. 2012), or functional molecules such as *Nrp-1* (Sarris, Andersen et al. 2008) or *LAG3* (Liang, Workman et al. 2008) in addition to the CAR although the number of accompanying genes which can be added is restricted by the size of the delivery vector (Cante-Barrett, Mendes et al. 2016).

Although adoptive Treg therapy is known to alleviate graft rejection, the exact mechanisms as to how this is achieved are not fully understood. This particularly relates to the location at which Tregs inhibit alloimmune responses. Various studies have demonstrated that Tregs suppress the activation of alloreactive T cells in draining lymph nodes associated with the allograft (Lee, Moore et al. 2006, Zhang, Schroppel et al. 2009) and that the ability to interact with DCs is of particular importance (Tadokoro, Shakhar et al. 2006, Tang, Adams et al. 2006, Koutrolos, Berer et al. 2014). However, this is contrary to the data shown in Chapter V and studies which have shown that re-transplanted allografts are not rejected when they are derived from tolerised mice (Hall, Jelbart et al. 1984, Graca, Cobbold et al. 2002). If alloresponses are principally inhibited in the draining lymph nodes, this would counter the argument that donor MHC class I-specific CAR Tregs facilitate the generation of an intragraft immunoregulatory milieu. Instead, this would suggest that the CAR Tregs mediated their protective capacity by engaging donor-derived and cross-dressed DCs, as was suggested for Tregs with direct allospecificity by Sagoo *et al*. (Sagoo, Ali et al. 2011). Through this mechanism, CAR Tregs may potentially inhibit both the activation of T cells with direct and indirect allospecificities, whereby the latter is achieved through linked suppression. Longitudinal imaging studies of transferred Tregs would resolve this conundrum and help address whether CAR Tregs alleviated graft rejection by suppressing Tconvs within the graft, or whether this was achieved by inhibiting T cell activation via the direct/semi-direct pathways in the lymph nodes (Sharif-Paghaleh, Sunassee et al. 2011). Indeed, similar studies performed by Parente-Pereira *et al*. in a cancer setting suggested that the majority of CAR T cells do not migrate to the tumour and instead, these cells remain in the liver, spleen and lymph nodes (Parente-Pereira, Burnet et al. 2011). Current work performed by Dr Nia Emami-Shahri and Dr Sophie Papa (Division of Cancer Studies, King's College London) aims to expand on these findings but in light of these results, the exogenous expression of specific homing receptors to promote preferential migration to the tumour site may be worthwhile (Kershaw, Wang et al. 2002, Di Stasi, De Angelis et al. 2009).

The principle of CAR Tregs can also be applied to treat various autoimmune diseases, particularly in light on ongoing clinical trials which are investigating the safety and efficacy of polyclonal Treg therapy in for the treatment of T1D (NCT01210664) (Bluestone, Buckner et al. 2015), lupus

erythematosus (NCT02428309) and uveitis (NCT02494492). This concept has already been exploited for the treatment of colitis (Elinav, Waks et al. 2008, Elinav, Adam et al. 2009, Blat, Zigmond et al. 2014) and MS (Fransson, Piras et al. 2012), where CAR Tregs were shown to inhibit undesired immune responses in specific tissues. As such, one may speculate that the concept of CAR Tregs can be applied for treating various autoimmune conditions where known autoantigens are presented. Investigating the efficacy of CARs that incorporated sequences from autoreactive antibodies would be of particular interest. Potential targets include glutamic acid decarboxylase (GAD)65, acetylcholine receptor and citrullinated proteins which are typically targeted by autoreactive antibodies in T1D, myasthenia gravis and RA patients, respectively (Lindstrom, Seybold et al. 1976, Vossenaar and van Venrooij 2004, Towns and Pietropaolo 2011, Kasagi, Zhang et al. 2014, Sinmaz, Nguyen et al. 2016). Research has also been conducted to investigate whether transplant rejection and autoimmunity can be reduced using CAR Tconvs. Specifically, these studies have used adaptations of CARs to redirect Tconvs towards lymphocytes which are responsible for eliciting undesired immune responses. In a transplant context, CAR T cells were generated which specifically killed $CD8^+$ T cells with direct allospecificity (Nguyen, Moisini et al. 2003). This was achieved by using a CAR that presented allogeneic MHC class I components. More recently, a similar approach was employed to generate CAR T cells which targeted autoreactive B cells. In this study, the CAAR presented elements of an autoantigen (Dsg-3) which, when naturally targeted by autoreactive antibodies, leads to the rare autoimmune blistering disease pemphigus vulgaris (Ellebrecht, Bhoj et al. 2016). B cells which engaged these Dsg3-derived elements through their BCR were subject to the cytotoxicity of the CAR T cell. As such, it is clear that CARs are versatile tools which can be used outside of their traditional cancer setting to treat transplant rejection and a variety of autoimmune conditions using a range of different approaches.

7.2.1 Considerations for applying CAR Tregs in the clinic

The fact that both Treg therapy and CAR technology are currently being investigated clinically suggest that the concept of CAR Tregs can be efficiently translated to the clinic. However, in addition to the issues discussed above, there are three key factors which must be considered before attempting this. The first of these is the fact that Tregs will need to be isolated with a high purity. The isolation of GMP-grade Tregs with a high purity is possible through the use of cell sorting outside of the UK and is currently employed in trials which are investigating the safety and efficacy of Treg therapy using Tregs with direct allospecificity (Table 1-1). This purity is of

importance as Tconvs with donor-MHC class I specificity would have a particularly deleterious effect following transfer.

The second consideration is the fact that it will be necessary to generate a library of CARs which recognise different HLA class I molecules. This would be a substantial undertaking and would be complicated by the CAR immunogenicity issues described above. The use of antigen-recognition domains which cross-react with various HLA subtypes, such as that which was used to generate the A2 CAR (Chapter III), would help limit the number of CARs which would need to be generated.

The final consideration which must be addressed before CAR Tregs can be applied in the clinic is the safety concerns which surround the use of genetically modified cells. As previously alluded to, these concerns exist due to the fact that retroviruses integrate their proviral DNA into the genome in a partially random manner which has the potential to activate oncogenes and cause the development of cancerous cell (section 1.3). Various strategies such as zinc-finger nucleases and TALENs have been investigated as potential strategies for inserting exogenous genes at specific genomic loci. One such approach which has gained popularity over the past two years is the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system. Using approaches which insert exogenous genes into genomic DNA in a regulated and specific manner would negate many of the safety concerns which surround genetic modification of cell therapy products.

The CRISPR/Cas9 system relies on two key molecules: i) a guide RNA, and; ii) the CRISPR associated protein (Cas)9 endonuclease. In isolation, this system utilises the guide RNA, which is typically ~20 nucleotides long, to direct the Cas9 endonuclease to a specific region of the genome which is complementary to the guide RNA. When this region is recognised, the Cas9 enzyme performs a double strand break of the DNA. Usually, this results in the host DNA repair system being activated whereby the DNA is re-ligated through non-homologous end joining (NHEJ), typically an imperfect process which yields nucleotide mutations such as insertions or deletions (indel) in this region of the genome. However, if a desired "DNA repair template" with flanking homology to this region of the genome is supplied, such as a desired GOI, this DNA fragment will be ligated into the broken DNA as part of the homology directed repair (HDR) system, resulting in insertion of the desired GOI into this specific region of the host DNA. Given the fact that the CRISPR/Cas9 gene delivery approach remains in its infancy, the efficiency with which target cells are successfully manipulated currently remains low (<10%). However, with this approach being adopted by numerous research groups over the past two years, improvements will doubtlessly occur rapidly. As such, it is likely that the CRISPR/Cas9 system will play an important role in future clinical trials which feature genetic modification.

7.3 CONCLUSION

Polyclonal Treg therapy is currently being investigated clinically as a means of limiting graft rejection and reducing the requirement of life-long drug-based immunosuppression. However, to avoid the risk of pan-immunosuppression and provide a tailored therapy, the successful generation and expansion of alloantigen-specific Tregs is required. The results presented in this thesis demonstrated that clinically-applicable CAR technology may be used to generate donor MHC class I-specific Tregs which suppress alloimmune responses more effectively than polyclonal Tregs. Certain issues remain to be addressed including whether CAR Tregs are superior to Tregs with direct/indirect allospecificity at alleviating graft rejection but taken together, this thesis suggests a future direction for Treg therapy and has important implications in the pursuit of tolerance in transplantation and autoimmunity.

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SUPPLEMENTARY DATA

A2 CAR-eGFP gene sequence

ATGGCCCTGCCCGTGACCGCCCTGCTGCTGCCCCTGGCCCTGCTGCTGCACGCCGCCCGGCCTCAGGTGCAGCTGGTGCAG
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Human αCD8 leader sequence

anti-HLA-A2 scFv sequence

9e10 epitope

hCD28-derived sequences

hCD3-derived sequences

eGFP ORF

Primer recognition sequence for generating A2 ΔCAR-eGFP

Figure S1 | Nucleotide sequence of A2 CAR-eGFP gene with elements highlighted.

SFG_A2-28ζ vector sequence

ATGGCCCTGCCCGTGACCGCCCTGCTGCTGCCCCTGGCCCTGCTGCTGCACGCCGCCCGGCCTCAGGTGCAGCTGGTGCAGAGCG
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GGACCCGTGGTGGAACTGACGAGTTCGGAACACCCGGCCGCAACCCTGGGAGACGTCCCAGGGACTTCGGGGGCCGTTTTTGTGG
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AGCATCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTCTGTATTTGTCTGAAAATATGGGCCCGGGCTAGACTGTTACCACTCCCT
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TCTACTTAGTCCAGCACGAAGTCTGGAGACCTCTGGCGGCAGCCTACCAAGAACAACTGGACCGACCGGTGGTACCTCACCCTTA
CCGAGTCGGCGACACAGTGTGGGTCCGCCGACACCAGACTAAGAACCTAGAACCTCGCTGGAAAGGACCTTACACAGTCCTGCTG
ACCACCCCCACCGCCCTCAAAGTAGACGGCATCGCAGCTTGGATACACGCCGCCCACGTGAAGGCTGCCGACCCCGGGGGTGGAC
CATCCTCTAGACTGCC

A2 CAR-eGFP sequence

Ampicillin resistance

Figure S2 | Nucleotide sequence of SFG retroviral vector with A2 CAR and ampicillin resistance ORFs highlighted.

pLNT/SFFV_A2-28ζ-eGFP vector sequence

ACCGCCATGGCCCTGCCCGTGACCGCCCTGCTGCTGCCCCTGGCCCTGCTGCTGCACGCCGCCCGGCCTCAGGTGCAGCTGGTGC
AGAGCGGCGGCGGCGTGGTGCAGCCCGGAGGCAGCCTGAGGGTGAGCTGCGCCGCCAGCGGCGTGACCCTGAGCGACTACGGCAT
GCACTGGGTGCGGCAGGCTCCCGGCAAGGGCCTGGAGTGGATGGCCTTCATCCGGAACGACGGCAGCGACAAGTACTACGCCGAC
AGCGTGAAGGGCCGGTTCACCATCAGCCGGGACAACAGCAAGAAGACCGTGAGCCTGCAGATGAGCAGCCTGCGGGCTGAGGACA
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AAGCGGCACCGACTTCACCTTCACCATCAGCAGCCTGCAGCCTGAGGACATCGCCACCTACTACTGCCAGCAGTACGACAACCTG
CCTCCCACCTTCGGCGGAGGCACCAAGCTGACCGTGCTGGGCGCGGCCGCCATCGAGGTGGAGCAGAAGCTGATCAGCGAGGAGG
ACCTGCTGGACAACGAGAAGAGCAACGGCACCATCATCCACGTGAAGGGCAAGCACCTGTGCCCCAGCCCCCTGTTCCCCGGCCC
CAGCAAGCCCTTCTGGGTGCTGGTGGTGGTGGGCGGCGTGCTGGCCTGCTACAGCCTGCTGGTGACCGTGGCCTTCATCATCTTC
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GCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGAC
CCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCT
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CACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCGAAACGCGTGGAGGTGGCGCCACCATGGTGAGCAAGGGCGAGGAG
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AGATGTTTCCAGGCTCCCCCAAGGACCTGAAATGACCCTGCGCCTTATTTGAATTAACCAATCAGCCTGCTTCTCGCTTCTGTTC
GCGCGCTTCTGCTTCCCGAGCTCTATAAAAGAGCTCACAACCCCTCACTCGGCGCGCCAGTCCTCCGACAGACTGAGTCGCCCGG
GGGGGATCC

A2 CAR-eGFP sequence

eGFP ORF

Ampicillin resistance

Figure S3 | Nucleotide sequence of pLNT/SFFV lentiviral vector with A2 CAR, eGFP and ampicillin resistance ORFs highlighted.

K^d CAR-eGFP sequence

ATGGCCAGCCCTCTCACCCGGTTTCTGAGCCTCAACCTGCTGTTGTTGGGTGAGTCCATAATCCTTGGTAGCGGAGAGGCTCAGG
TGCAGCTGCAGCAGTCAGGGGCCGTTCTCGTGAAGCCTGGCGCAAGCGTGAAAATTTCCTGCAAGGTGTCTGGCTACGCTTTTTC
AATATACTGGATGAACTGGGTCAAGCAGCGACCTGGCAAGGGATTGGAATGGATCGGCCAGATATTTCCTGGGGACGACGACACC
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CCAGTGAAGACAGCGCTGTGTACTTTTGTGCAAGTGGGCCAATTGGTAAGGGATTTGCCTACTGGGGACAGGGCACTCTGGTGAC
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AGCTACCTTGCCGCATCTCCTGGAGAAACAATCATTATTAATTGTAGAGCATCCAAATCCATTAGCCGGTACTTGGCTTGGTACC
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TACCCATACACATTTGGAGGAGGCACCAAGCTTGAGATTAAGGTGATCATTATTGAATTT**GAACAGAAGCTCATATCGGAGGAGG**
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GCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGC
TGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGA
GCTGTACAAGTAA

Mouse αCD8 leader sequence	anti-K^d scFv sequence
9e10 epitope	mCD28-derived sequences
mCD3-derived sequences	eGFP ORF

Figure S4 | Nucleotide sequence of K^d CAR-eGFP gene with elements highlighted.

pMP71-PRE_K^d-28ζ-eGFP vector sequence

GCCACCATGGCCAGCCCTCTCACCCGGTTTCTGAGCCTCAACCTGCTGTTGTTGGGTGAGTCCATAATCCTTGGTAGCGGAGAGG
CTCAGGTGCAGCTGCAGCAGTCAGGGGCCGTTCTCGTGAAGCCTGGCGCAAGCGTGAAAATTTCCTGCAAGGTGTCTGGCTACGC
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K^d CAR-eGFP sequence	eGFP ORF
PRE sequence	Ampicillin resistance

Figure S5 | Nucleotide sequence of pMP71-PRE retroviral vector with K^d CAR, eGFP and ampicillin resistance ORFs highlighted.

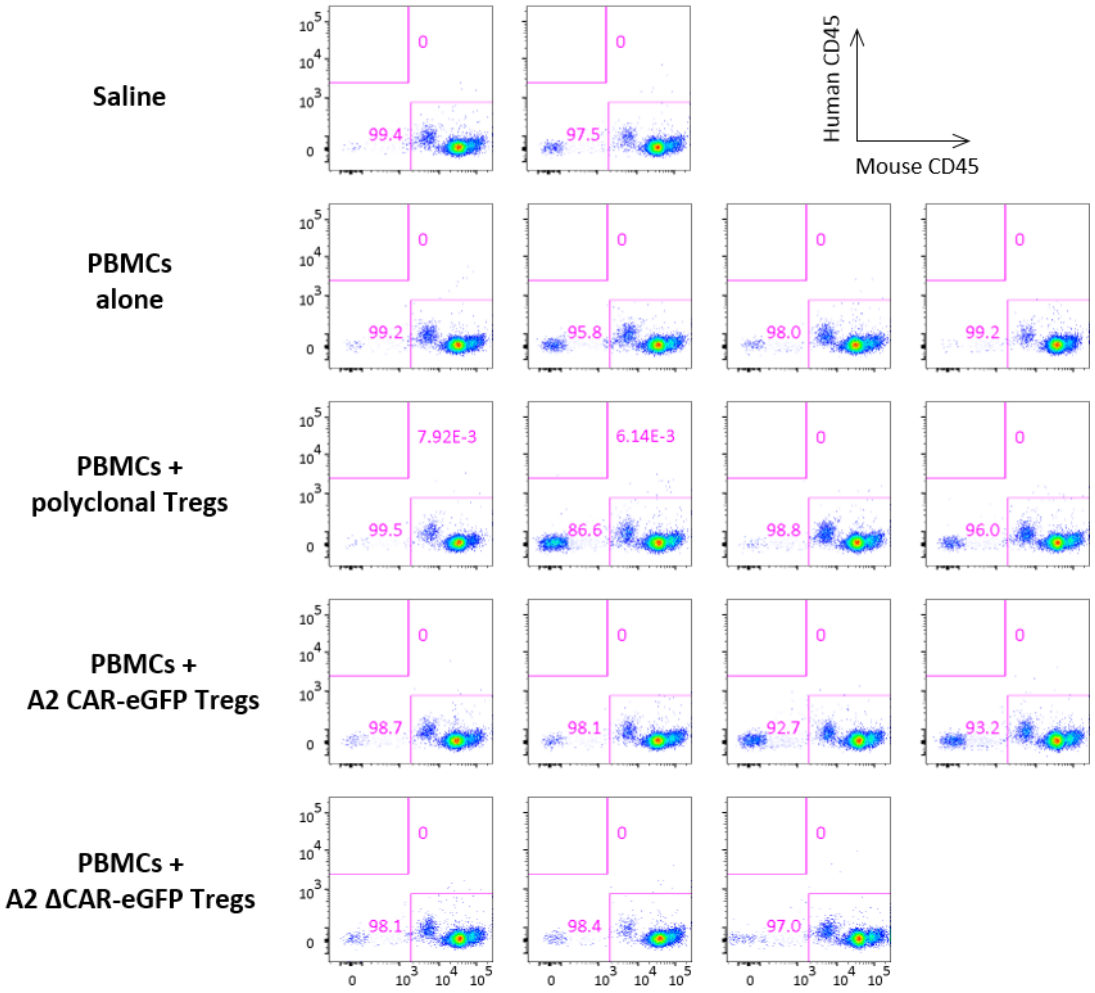

Figure S6 | **Engraftment of Ch1-2hSa mice.** *The proportion of human cells present in the spleens of Ch1-2hSa mice was measured 100 days following transfer of 5x10^6 PBMC ± 1x10^6 Tregs iv. 5x10^5 splenocytes were stained with fluorescently conjugated antibodies specific for mouse and human CD45 and expression of these markers was measured by flow cytometry.*

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Antigen-specificity using chimeric antigen receptors: the future of regulatory T-cell therapy?

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Abstract

Adoptive regulatory T-cell (Treg) therapy using autologous Tregs expanded *ex vivo* is a promising therapeutic approach which is currently being investigated clinically as a means of treating various autoimmune diseases and transplant rejection. Despite this, early results have highlighted the need for potent Tregs to yield a substantial clinical advantage. One way to achieve this is to create antigen-specific Tregs which have been shown in pre-clinical animal models to have an increased potency at suppressing undesired immune responses, compared to polyclonal Tregs. This mini review outlines where Treg therapy currently stands and discusses the approaches which may be taken to generate antigen-specific Tregs, including the potential use of chimeric antigen receptors (CARs), for future clinical trials.

Regulatory T-cells

Regulatory T-cells (Treg) are a subpopulation of T-cells which play a critical role in the maintenance of tolerance *in vivo* [1,2]. The idea of regulatory immune cells was first proposed by Gershon and Kondo in 1970 [3], at which time they were termed 'suppressor T-cells'. However, in the face of scepticism [4], research on these cells diminished until 1995 when Sakaguchi et al. [5] published a seminal paper describing a population of $CD4^+CD25^+$ T-cells which were capable of inhibiting autoimmunity upon adoptive transfer (described below). This was followed by a series of papers which went on to demonstrate that murine $CD4^+CD25^+$ T-cells were a unique lineage of T-cells [6] which were capable of suppressing undesired immune responses in a plethora of different contexts ranging from organ transplantation [7–12], where a clear correlation between the Treg numbers in a graft and allograft survival has been made [13,14], to various autoimmune diseases [15–19].

Key words: adoptive cell therapy, antigen-specific, autoimmune disease, chimeric antigen receptor, regulatory T-cell, transplantation.

Abbreviations: APC, antigen presenting cell; BMT, bone marrow transplantation; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CTLA, cytotoxic T-lymphocyte antigen; EAE, experimental autoimmune encephalomyelitis; FACS, fluorescence-assisted cell sorting; FOXP3, forkhead-box protein 3; GITR, glucocorticoid-induced tumour-necrosis factor-receptor-related protein; GMP, good manufacturing practice; GvHD, graft-versus-host disease; HLA, human leucocyte antigen; IL, interleukin; IPEX, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; pTreg, periphery-derived regulatory T-cell; RA, rheumatoid arthritis; scFv, single-chain variable fragment; T1D, Type I diabetes; TALEN, transcription activator-like effector nuclease; TCR, T-cell receptor; Teff, effector T-cell; TGF, transforming growth factor; Th3, T helper 3 cell; TM, transmembrane; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumour necrosis factor; TNP, 2,4,6-trinitrophenol; Tr1, Type-I regulatory T-cell; Treg, regulatory T-cell; tTreg, thymus-derived regulatory T-cell.

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It is now well-established that many types of Treg exist. The best characterized are the naturally occurring Tregs which develop in the thymus (tTreg). tTregs constitute approximately 5–10% of all peripheral $CD4^+$ T-cells [9,15] and are characterized by a high expression of the fundamental transcription factor forkhead-box protein 3 (FOXP3) [1]. Mutations in the *FoxP3* gene have been directly linked with the severe autoimmune disease immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), accentuating the critical role of $FOXP3^+$ Tregs *in vivo* [1]. tTregs are also characterized in humans by a high expression of CD4, the interleukin (IL)-2 receptor α-chain, CD25, and a low expression of the IL-7 receptor α-chain, CD127 [14]. However, other markers may be used to characterize human tTregs including CD62L, CD27, CD45RA, human leucocyte antigen (HLA)-DR, glucocorticoid-induced tumour-necrosis factor (TNF)-receptor-related protein (GITR), CD73, CD39 and cytotoxic T-lymphocyte antigen (CTLA)-4 [14]. In the periphery, Tregs are capable of suppressing a wide range of target cells including $CD4^+$ and $CD8^+$ effector T-cells (Teff), B-cells, natural killer cells and antigen presenting cells (APCs) [2]. To accomplish this, Tregs employ a range of contact-dependent and contact-independent mechanisms [2,15] such as the CTLA-4-facilitated removal of co-stimulatory molecules from APCs [20] or the secretion of inhibitory factors including exosomes [21] and inhibitory cytokines [2].

Another Treg population of interest is the induced Tregs which are derived in the periphery following antigen exposure (pTreg) [22]. Although not as well characterized as tTregs, pTregs have been implicated with the induction and maintenance of tolerance *in vivo*, mostly through the

secretion of immunosuppressive cytokines. It is believed that at least two types of pTregs exist, namely Type-I regulatory T-cells (Tr1) and T helper 3 cells (Th3) which secrete high levels of IL-10 and transforming growth factor (TGF)-β, respectively [23]. A feature of particular interest is the fact that pTregs may be generated from naïve T-cells *in vitro*, a phenomenon which has been applied to generate Tr1 cells for clinical application in Crohn's disease [24] (detailed below).

Regulatory T-cell therapy – current applications

Due to their ability to regulate immune responses, the adoptive transfer of *ex vivo*-expanded Tregs has emerged, clinically, as an exciting therapeutic strategy for suppressing undesired immune responses, both in the context of transplantation and autoimmunity. Unlike drug-based immunosuppressive regimens which have detrimental effects on the patient's wellbeing [25] and do not offer a complete treatment option [14,23], Treg therapy has the potential to be a highly personalized technique which allows for the manipulation of a patient's immune system in order to promote immunological tolerance. The essence of what Treg therapy entails can be split into three key steps: cell isolation, *ex vivo* expansion and subsequent re-infusion of Tregs (Figure 1A).

The potential for Treg therapy using $CD4^+CD25^+$ tTregs was initially demonstrated by Sakaguchi et al. in 1995 [5] in a study which showed that the onset of autoimmune diseases in nude mice injected with $CD4^+CD25^-$ T-cells was hindered by the co-injection of $CD4^+CD25^+$ T-cells. Furthermore, they showed prolonged survival of skin allografts following adoptive transfer of $CD4^+CD25^+$ T-cells. Following on from this seminal paper, various groups demonstrated the potential for $CD4^+CD25^+$ Treg therapy in a variety of animal models including graft-versus-host disease (GvHD) [26], organ transplantation [7], multiple sclerosis (MS) [16], colitis [17], lupus erythematosus [18], Type I diabetes (T1D) [15] and rheumatoid arthritis (RA) [19].

The success of these animal-based studies led to an interest in utilizing Tregs as a potential therapy in humans. As such, various groups developed protocols for isolating and expanding autologous human Tregs [14,23,27]. However, the lack of a definitive human Treg marker and the requirement for compliance with good manufacturing practice (GMP) has meant that Treg therapy is only now moving from the bench to the clinic [14,23,27]. To date, a handful of clinical trials have demonstrated that Treg therapy, using autologous Tregs specific for self-antigens (self-specific) and expanded in a polyclonal manner using anti-CD3/CD28 beads, is safe and potentially efficacious with some T1D patients being insulin-independent for at least one year following Treg infusion [28,29]. However, in the context of GvHD following bone marrow transplantation (BMT) [30–33], early results question the efficacy of these cells as a therapeutic option. In some cases, patients with GvHD were successfully withdrawn from immunosuppression without evidence of recurrence [32]. However, approximately half of all patients with GvHD did not survive, despite Treg treatment [30,33]. As such, it is clear that further research to increase the potency or specificity of these cells is required.

Currently, several clinical trials, listed on www.clinicaltrials.gov, are investigating the safety and/or efficacy of polyclonal Tregs in the contexts of kidney transplantation (The ONE Study: NCT02129881/NCT02371434/NCT02091232, TASK: NCT02088931, NCT02145325 and NCT01446484), liver transplantation (ThRIL: NCT02166177 and NCT01624077), GvHD following BMT (TREGeneration: NCT02385019, NCT01903473, NCT01937468, NCT01911039, NCT00602693 and NCT02526329), T1D (NCT01210664), lupus erythematosus (NCT02428309) and uveitis (NCT02494492).

As alluded to above, one possible way to improve the efficacy of Treg therapy is to use Tregs with a defined antigen-specificity. This is supported by a substantial number of studies conducted in pre-clinical animal models which have demonstrated that Tregs specific for a desired antigen are functionally superior to polyclonal Tregs [9–12,15,26,34]. In 2004, Tang et al. [15] demonstrated that development of T1D induced by the transfer of $25x10^6$ diabetogenic cells could be blocked by the transfer of $2x10^6$ Tregs specific for islet β-cell antigens, but not as efficiently blocked by the transfer of $8x10^6$ self-specific Tregs, suggesting that the antigen-specific Tregs were over four times more potent at preventing disease manifestation. Furthermore, these antigen-specific Tregs could not only prevent the development of T1D but were also able to reverse diabetes following disease onset, further accentuating the potency of these cells for clinical use.

Approaches to generate regulatory T-cells with antigen-specificity

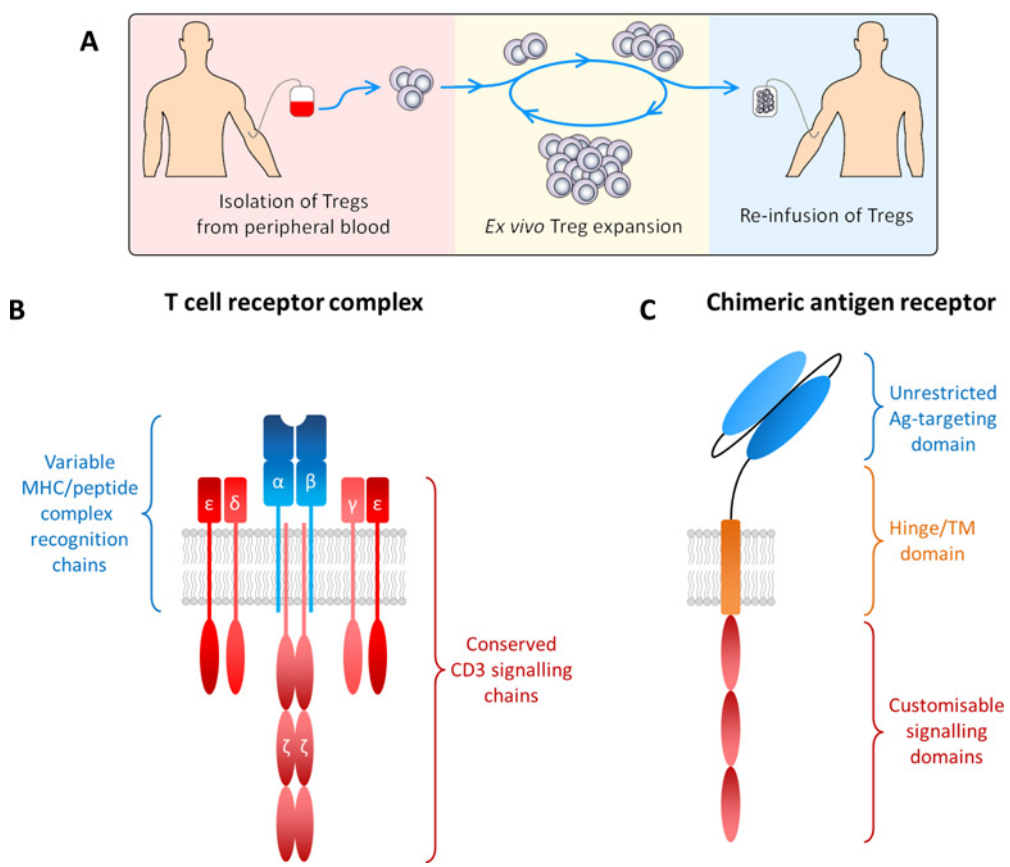
Preferential expansion using antigen presenting cells

To date, we are aware of only one clinical trial (NCT02327221) which has investigated the potential use of antigen-specific Tregs for therapy in the context of Crohn's disease [24]. In this trial, ovalbumin (OVA)-specific Tr1 cells were generated *ex vivo* by expanding peripheral blood mononuclear cells (PBMC) using modified Drosophila Schneider 2 feeder cells as artificial APCs, thus preferentially selecting cells which expressed a T-cell receptor (TCR) α and β chain with OVA-specificity (Figure 1B). Adoptive transfer of these Tr1 cells resulted in 8 out of 20 patients showing significant signs of remission, as defined by a reduction in Crohn's Disease Activity Index.

Another context in which the potential for antigen-specific Tregs is currently being assessed clinically is in the field of transplantation as a means of limiting graft rejection. Graft rejection is primarily driven by alloreactive T-cells which recognize donor antigens via three distinct

Figure 1 | Schematic diagrams outlining the process of Treg therapy and the antigen-targeting moieties which may be used to redirect the specificity of Tregs

(**A**) To acquire an autologous population of Tregs which may be applied in a Treg therapy protocol, peripheral blood is drawn from the patient and Tregs are isolated either through the use of GMP-compatible fluorescence-assisted cell sorting (FACS) or *in vitro* negative and positive selection protocols using magnetic beads (red). The Tregs are then activated, usually in a polyclonal manner using anti-CD3/CD28 beads, and undergo a single or multiple rounds of stimulation in order to expand the number of Tregs *ex vivo* (yellow). This expanded population of Tregs is then infused back into the patient, typically as part of a combination therapy protocol (blue). (**B**) The antigen-specificity of Tregs is naturally determined by the α and β TCR genes which are expressed (blue). *In vitro*, Tregs can be genetically manipulated to express specific α and β TCR genes which are capable of signalling through the use of endogenous CD3 signalling chains (red), thereby conferring specificity towards a desired MHC-peptide complex. (**C**) Tregs may be artificially redirected towards an antigen of interest through the transduction and expression of a chimeric antigen receptor (CAR). CARs utilize an extracellular antigen-targeting domain (blue) which is typically derived from an antigen-specific antibody. As such, CARs are capable of binding their target antigen in an MHC-independent manner. Engagement of the antigen is translated into the activation of tailored intracellular T-cell signalling cascades via customisable intracellular TCR and co-stimulatory signalling domains (red). Ag: antigen, TM: transmembrane.

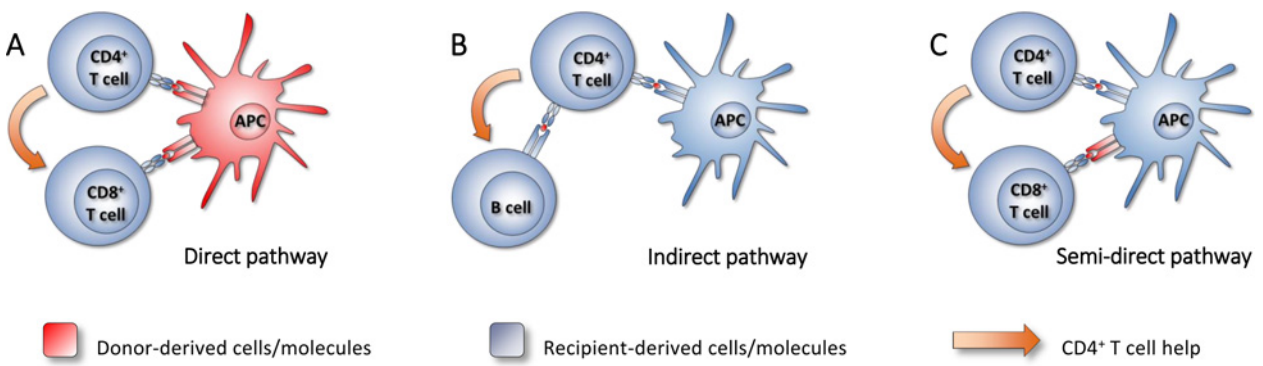


pathways of allorecognition. In the direct pathway, recipient T-cells recognize allopeptides presented in the context of allogeneic major histocompatibility complexes (MHC) by donor-derived APCs (Figure 2A). In the indirect pathway, recipient T-cells recognize allopeptides presented in the context of self-MHC by recipient APCs (Figure 2B). More recently, the semi-direct pathway was described in which recipient-derived APCs acquire and present intact donor MHC-peptide complexes. As such, these APCs are capable of presenting not only allopeptides in the context of recipient MHC (indirect pathway), but also intact donor MHC-peptide complexes (direct pathway) contributing to the activation of T-cells with either direct or indirect allospecificity (Figure 2C).

It has been demonstrated in various animal models that Tregs which possess the same allospecificity as the T-cells which drive graft rejection are far more effective at protecting from graft rejection than polyclonal Tregs. Murine Tregs with direct allospecificity have successfully been preferentially expanded by us [9,10] and others [34] using allogeneic APCs and have demonstrated a superior efficacy at protecting from allograft rejection *in vivo* compared to self-specific Tregs.

Figure 2 | Schematic diagram detailing the three distinct pathways of allorecognition

(**A**) In the direct pathway of allorecognition, donor-derived antigen presenting cells (APCs) migrate from the transplanted allograft to secondary lymphoid organs where they present donor antigen in the context of donor MHC molecules. Recipient-derived CD4$^+$ T-cells binding antigen presented by MHC class II are then capable of facilitating the activation of recipient-derived CD8$^+$ T-cells recognizing antigen presented by MHC class I. (**B**) In the indirect pathway of allorecognition, recipient-derived APCs infiltrate the transplanted allograft where they acquire donor antigen. These APCs then migrate to secondary lymphoid organs where they present this donor antigen in the context of recipient MHC class II to recipient-derived CD4$^+$ T-cells which can facilitate the activation of recipient B-cells. (**C**) In the semi-direct pathway of allorecognition, recipient-derived APCs infiltrate the transplanted allograft and uptake intact donor class I and class II MHC/peptide complexes. In addition, these APCs also uptake, process and present peptides derived from donor MHC molecules in the context of recipient MHC class II. Therefore, in the secondary lymphoid organs, recipient-derived APCs are capable of presenting donor antigen in the context of recipient MHC class II to recipient-derived CD4$^+$ T-cells and in the context of donor MHC class I to recipient-derived CD8$^+$ T-cells, enabling the CD4$^+$ T-cells to facilitate in the activation of the CD8$^+$ T-cells.



More recently, we confirmed these results using human allospecific Tregs expanded using allogeneic dendritic cells [12] and B-cells [11] as APCs. Employing a human skin xenograft transplant model, we demonstrated that allospecific Tregs were more potent than polyclonal Tregs at protecting skin allografts from injury induced by the transfer of allogeneic PBMCs. Following on from these promising results, the efficacy of direct allospecific Tregs at promoting transplant tolerance is due to be assessed in kidney (DART as part of The ONE study: NCT02244801) and liver transplant recipients (deLTa: NCT02188719 and NCT01624077).

In spite of the promising results detailed above, the expansion of Tregs with indirect allospecificity has proved more challenging due to the low frequency of these cells naturally present in the periphery. A solution to this issue is to genetically manipulate Tregs with self-specificity to express one of two antigen-targeting moieties: either a specific TCR complex or a chimeric antigen receptor (CAR), both options of which are discussed below (Figure 1 and Table 1).

T-cell receptors – a traditional approach to confer antigen-specificity

Antigen-specificity can be conferred on to Tregs through the expression of defined TCR α and β chain genes (Figure 1B). In animal models, this can be accomplished either by transducing self-specific Tregs to express exogenous α and β TCR genes which are specific for a desired antigen or by isolating Tregs from transgenic mice which express these TCR transgenes in place of rearranged α and β genes (Table 1). However, translating the successes of TCR gene modification into the clinic is hampered by the fact that Tregs inherently contain their own endogenous TCR genes. As such, both the endogenous and introduced α and β chains are co-expressed in the same cell with no preference as to which α and β chain is partnered together. As half of the TCR complexes on the cell surface will inevitably consist of a mixture of endogenous and transduced α and β chains, these receptors will not be specific for the antigen of interest.

Various strategies have been explored to reduce this issue. One such solution is to target Tregs with α and β TCR chains structurally modified in the constant region, such that they pair with endogenous chains with a low efficiency [35]. Alternatively, strategies to decrease the expression of endogenous α and β chain TCR genes have also been explored using either RNA-interference to knockdown endogenous TCR expression [36] or genome editing techniques such as transcription activator-like effector nucleases (TALENs) to partially knockout endogenous TCR genes [37]. With an ever-increasing number of solutions to this endogenous/exogenous TCR dimerization problem coming to light, research into the use of TCR genes to redirect the specificity of Tregs has prevailed. As such, the superior efficacy of TCR-modified antigen-specific Tregs, as compared to self-specific Tregs, has been demonstrated in mouse models for the treatment of colitis [38], MS [39],

Table 1 | **A non-exhaustive selection of studies in which antigen-specific Tregs have been isolated or generated and demonstrated to be efficacious *in vivo***

Setting	Targeting moiety	Specificity	Reference
Colitis	CAR	TNP/CEA	[47–49]
Multiple sclerosis	CAR	MOG	[50]
Autoimmune gastritis	TCR	H^+/K^+ ATPase proton pump	[40]
Colitis	TCR	OVA peptide presented by BALB/c MHC II	[38]
Multiple sclerosis	TCR	MBP	[39]
Rheumatoid arthritis	TCR	Type II collagen or OVA peptide presented by C57BL/7 MHC II	[41,42]
Type I diabetes	TCR	Unidentified islet β-cell antigen	[15,43]
GvHD following BMT	TCR	Direct allospecificity	[26,44]
Transplantation	TCR	Direct allospecificity	[8,11,12]
Transplantation	TCR	Indirect and dual allospecificity	[9,10,34]

autoimmune gastritis [40], RA [41,42], T1D [15,43], GvHD [26,44] and organ transplantation [8–12].

One of the first studies to compare the efficacy of antigen-specific and self-specific Tregs upon adoptive transfer *in vivo* was conducted by Taylor et al. [26] in the context of GvHD. In addition to being one of the first papers to explore the potential for Treg therapy using *ex vivo*-expanded Tregs, this study also demonstrated that murine Tregs preferentially expanded using allogeneic splenocytes were more effective at protecting from GvHD than Tregs expanded using anti-CD3, upon adoptive transfer.

In a transplant setting, although Tregs with direct allospecificity (Figure 2A) can be isolated through preferential expansion, as outlined above, acquiring Tregs with indirect allospecificity (Figure 2B), recognizing donor peptide presented in the context of recipient MHC molecules, is more complex due to the low frequency of these cells in recipients, prior to the transplant procedure. However, in 2008, we demonstrated in a mouse setting that Tregs with indirect allospecificity could be generated by transducing self-specific Tregs with specific TCR genes [10]. Furthermore, we [9] and others [34] have demonstrated that Tregs with dual direct and indirect allospecificity can prolong graft survival more effectively than any population of Tregs found naturally, highlighting the potential for gene-modified cell therapy in the clinic.

Chimeric antigen receptors – a novel approach to confer antigen-specificity

An approach which has been explored extensively in cancer research for generating antigen-specific T-cells is to transduce polyclonal T-cells with CARs [45] (Figure 1C). Adoptive transfer of effector T-cells expressing CARs specific for a wide range of antigens such as ErbB receptors or CD19 has successfully led to the treatment of various cancers in murine models [45]. These studies paved the way for several clinical trials which are designed to demonstrate the advantageous clinical applications of CAR-transduced T-cells for treating various cancers in humans, such as chronic lymphocytic leukaemia, non-Hodgkin's lymphoma and B-cell lymphoma (extensively reviewed by Maher [45]).

CARs contain an extracellular antigen-binding domain which typically comprises a single-chain variable fragment (scFv) capable of binding a specific antigen with high affinity in an MHC-independent manner (Figure 1C). This is linked via a transmembrane (TM) domain to a series of customised intracellular TCR and co-stimulatory signalling domains, allowing the CAR to signal in a similar manner to a TCR complex. The concept of using CARs to redirect the specificity of T-cells was first suggested by Eshhar and colleagues over 25 years ago [46]. The resultant first generation CARs which were developed at this time were engineered to mimic endogenous TCRs, thus contained a CD3ζ signalling domain. However, these CARs were found to have a low efficacy attributed to the lack of co-stimulatory domains. Therefore, second generation CARs were designed to incorporate a single co-stimulatory domain and third generation CARs were developed to incorporate multiple co-stimulatory domains from different sources, such as CD28, 4-1BB, OX40, Lck, inducible T-cell co-stimulator (ICOS), CD27, 2B4 and DAP10 [45].

CAR-technology is now emerging as an approach for redirecting the specificity of Tregs for the treatment of autoimmunity (Table 1).

In 2008, Elinav et al. [49] generated Tregs expressing a CAR specific for 2,4,6-trinitrophenol (TNP), a colitis-associated model antigen. In this study, Tregs were isolated from transgenic mice expressing the TNP-specific CAR under the CD2 promoter. Upon adoptive transfer, these Tregs prevented the development of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, but not oxazolone-induced colitis, highlighting the antigen-specific nature of these Tregs. Furthermore, *in vivo* imaging of the Tregs using fluorescence showed that these cells accumulated in the colon, suggesting that CAR Tregs traffic to sites where their cognate antigen is present. These authors also showed that self-specific Tregs retrovirally transduced to express the TNP-specific CAR were equally capable of preventing colitis [48] and that similar

results could be obtained through targeting different colitis-associated antigens [47].

The protective capacity of CAR Tregs has also been demonstrated for the treatment of MS. In 2012, Fransson et al. [50] transduced murine $CD4^+$ T-cells to express both a CAR specific for myelin oligodendrocyte glycoprotein (MOG) and the *FoxP3* gene, resulting in cells which elicited both a suppressive capacity and functioned in a MOG-dependent manner. These cells were found to localize to the central nervous system where they were capable of alleviating the symptoms of experimental autoimmune encephalomyelitis (EAE), following adoptive transfer.

Although the use of CARs to redirect the specificity of Tregs has been explored in the context of autoimmunity, we are aware of no publication which has demonstrated the efficacy of CAR Tregs in a transplant context. However, with the success CAR-expressing Tregs have had in protecting from autoimmunity by targeting an antigen expressed in the organ of interest, we believe similar results may be achieved in a transplant setting by targeting donor MHC molecules. These ideas are currently under investigation.

Conclusions

Recent developments and advancements of techniques allowing for the isolation and expansion of GMP-grade Tregs have progressed Treg therapy from a vision to a reality. However, to escalate Treg therapy to a preferential treatment option, the development of GMP-compatible approaches for increasing the potency of Tregs *in vivo* is required. The use of antigen-specific Tregs is believed by many to be a solution to improve the efficacy of Tregs *in vivo*, as demonstrated by the implementation of clinical trials now investigating the efficacy of antigen-specific Tregs in the contexts of autoimmunity and transplantation. We believe that gene modification for the generation of antigen-specific Tregs is the next logical step for Treg therapy.

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What Is Direct Allorecognition?

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Abstract Direct allorecognition is the process by which donor-derived major histocompatibility complex (MHC)-peptide complexes, typically presented by donor-derived 'passenger' dendritic cells, are recognised directly by recipient T cells. In this review, we discuss the two principle theories which have been proposed to explain why individuals possess a high-precursor frequency of T cells with direct allospecificity and how self-restricted T cells recognise allogeneic MHC-peptide complexes. These theories, both of which are supported by functional and structural data, suggest that T cells recognising allogeneic MHC-peptide complexes focus either on the allopeptides bound to the allo-MHC molecules or the allo-MHC molecules themselves. We discuss how direct alloimmune responses may be sustained long term, the consequences of this for graft outcome and highlight novel strategies which are currently being investigated as a potential means of reducing rejection mediated through this pathway.

Keywords Transplantation · Allospecificity · Allorecognition · Multiple binary complexes · High determinant density

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Introduction

The ability of immune cells to distinguish between 'self' and 'non-self' is of fundamental importance. It ensures that invading pathogens are efficiently removed whilst tolerance towards cells of self-origin is maintained. In transplantation, the introduction of 'non-self' cells or tissues into a recipient can trigger an immune response. This is initiated when antigens derived from a genetically distinguishable member of the same species are recognised as foreign, a process termed 'allorecognition'. Subsequent immune cell activation and elicitation of an immune response directed towards alloantigen-expressing cells ultimately results in graft-versus-host disease (GvHD) following bone marrow transplantation (BMT) or graft rejection following solid organ transplantation.

Organ transplantation is inherently an invasive surgical approach which is inevitably accompanied by ischemia/reperfusion injury, inflammation and tissue damage [1]. Consequently, innate immune responses such as the complement cascade [2, 3] are initiated which contribute to graft rejection [4]. However, studies conducted in neonatally thymectomised [5] and irradiated adult [6–8] mice have demonstrated that the most deleterious immune responses are driven by recipient-derived T cells. These cells have been

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described to recognise alloantigens via three pathways of allorecognition: the direct, indirect and semi-direct pathways. The direct pathway is initiated by donor-derived antigen-presenting cells (APC) which present allogeneic major histocompatibility complex (MHC)-peptide complexes to recipient T cells. Conversely, the indirect pathway relies on recipient-derived APCs which uptake, process and present allopeptides in the context of self-MHC class II. More recently, the semi-direct pathway was described in which recipient-derived APCs present both acquired, intact allo-MHC-peptide complexes (direct) and allopeptides in the context of self-MHC (indirect). In this review, we focus on the direct and semi-direct pathways of allorecognition.

Premise of Direct Allorecognition

The unusual strength and vigour of direct alloimmune responses was first demonstrated by Bain et al. [9] through the use of in vitro-mixed leukocyte reactions (MLR). It was discovered that mixing leukocytes from two genetically unique individuals resulted in significant leukocyte activation, a phenomenon which was not observed by mixing leukocytes from genetically identical individuals. Subsequent in vivo studies demonstrated that similarly aggressive immune responses were observed in rodents which received allogeneic transplants [10]. This vigorous response was attributed to the presence of donor-derived 'passenger' leukocytes which were co-transferred into the recipient during the transplant procedure. Depletion of these cells from thyroid [11] or pancreatic [12] allografts, achieved by culturing the allografts in vitro to facilitate passenger leukocyte egression, resulted in a prolonged graft survival. In the former study, this prolongation was reversed by the infusion of donor peritoneal exudate cells (PEC), suggesting that recipient T cells with direct allospecificity must be activated by donor-derived APCs in order to destroy transplanted allografts [11].

Subsequent investigations performed by Lechler and Batchelor [13, 14] demonstrated that the principle 'passenger' leukocytes responsible for activating recipient T cells were dendritic cells (DC). In these studies, rat kidneys were 'parked' in intermediate recipients to deplete passenger leukocytes, prior to engraftment in a terminal recipient. The outcome was prolonged allograft survival which was prevented by the repletion of donor DCs, implicating a significant role for these cells in acute allograft rejection. Additional studies proceeded to suggest that these DCs prime and activate recipient T cells in secondary lymphoid tissues [15, 16].

Models Explaining Direct Allorecognition

The strength and vigour with which direct alloimmune responses are elicited may be explained by the fact that all individuals have a high-precursor frequency of T cells specific for allogeneic MHC-peptide complexes. Approximately 0.01 % of the cells in a standard T cell repertoire are capable of responding to a specific foreign peptide presented by a self-MHC molecule. However, 1–10 % of these T cells can engage intact foreign MHC-peptide complexes (direct allorecognition) [17•]. Two models have been proposed to account for this unusually high frequency of T cells with direct allospecificity, each of which places an emphasis on the different components which comprise an MHC-peptide complex: the allopeptide and the allo-MHC molecule.

Peptide-Centric Model

The first model focuses on the contribution of the allopeptide bound in the groove of the allo-MHC. It is believed that specific structural components of self-MHC molecules are 'mimicked' by allo-MHC molecules. As such, self-restricted T cells dock and make contacts with allo-MHC molecules in the same manner as they would with self-MHC molecules. However, the binding groove of the self- and allo-MHC molecules is vastly different, thus the peptides presented by each is significantly different, despite being derived from similar endogenous proteins.

Given the random nature with which TCRs are genetically rearranged, a standard repertoire comprises T cells with a wide spectrum of specificities. As such, the recognition frequency of a self-MHC presenting a specific foreign peptide is low: often, a very small proportion of T cells engage a specific MHC-peptide complex. However, in this model, it is not one foreign peptide which is presented by allo-MHC molecules but instead, an entire pool of foreign peptides thus donor-derived cells will activate a variety of recipient T cells with a range of specificities. This hypothesis was initially proposed by Matzinger and Bevan in 1977 and is termed the 'multiple binary complexes' hypothesis (Fig. 1a) [18].

In 1988, Eckels et al. [19] demonstrated the importance of allopeptides in the activation of T cells with direct allospecificity. In this study, HLA-DR1-restricted alloreactive T cell clones were co-cultured with allogeneic APCs in the presence and absence of a competing peptide. T cell proliferation induced by the presentation of allopeptides in the context of HLA-DR1 was abrogated when the allopeptides were displaced by competing influenza haemagglutinin-based peptides. Subsequently, Panina-Bordignon et al. [20] demonstrated that APC presentation of peptides derived from endogenous proteins contributes significantly to the activation of alloreactive T cell clones. Of 1489 $CD4^+$ T cell clones analysed, 6.6 % specifically responded to APCs which presented human serum albumin (HSA)-derived peptides but not foetal calf serum (FCS)-derived peptides, despite the peptides being presented by the same HLA-DR molecule. Conversely, it has been shown that presentation of allo-MHC molecules lacking allopeptides, achieved through the use of MHC mutants [21] or acid-treatment of target cells [22], triggers a limited response from alloreactive T cells. In the latter study, T

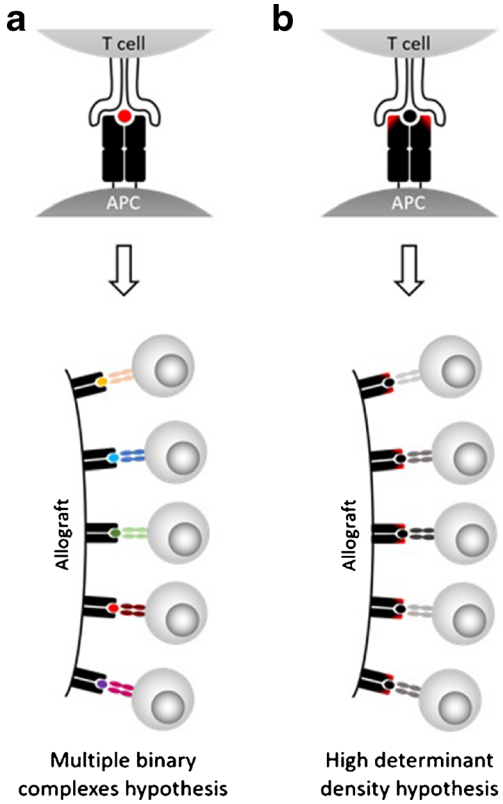

Fig. 1 Comparison of the two principle theories explaining the high frequency of T cells with direct allospecificity. **a** Multiple binary complexes hypothesis. The elements of the allogeneic MHC molecule which interact with the TCR mimic those which are found in self-MHC molecules. As such, it is the presence of the allopeptide (*red*) which drives recognition of the allogeneic MHC-peptide complex. Allograft presentation of various allopeptides in the contexts of MHC molecules which are perceived as 'self' results in the activation of a range of T cells, each expressing a TCR specific for a different MHC-peptide complex. **b** High determinant density hypothesis. Structural differences in the polymorphic regions of the allo-MHC molecule are detected by the TCR (*red*). The high density of cognate allo-MHC molecules which possess these polymorphisms on donor-derived APCs facilitates the efficient activation of recipient T cells which recognise the allogeneic MHC molecule with a low, medium or high affinity

cell responses were restored by the addition of synthetic allopeptides. Furthermore, various groups have described allopeptide sequence consensuses [23] and have demonstrated that through disrupting the binding of these specific allopeptides, there is a loss of response from alloreactive T cells [24]. More recent studies have employed point mutation approaches to determine which motifs in a TCR are important for eliciting T cell activation and have concluded that TCR-peptide interactions are fundamental in direct allorecognition [25].

MHC-Centric Model

The second model focuses on the fact that allo-MHCs are structurally different to self-MHCs. Whilst the majority of elements recognised by a TCR are conserved across various MHC subtypes [26], specific amino acid polymorphisms present in the allo-MHC molecule may modify the manner by which a self-restricted TCR docks with an MHC, irrespective of the peptide presented.

In this model, it is suggested that fundamental differences exist between self- and allo-MHCs in specific polymorphic residues which are exposed to potential docking TCRs. These residues cause the MHC-peptide complex to be recognised as foreign, thus the peptide presented stabilises the MHC-peptide complex but has little influence in the recognition process. Additionally, the affinity with which this TCR:allo-MHC interaction occurs may have implications in the alloresponse observed. Whilst T cells are selected to bind self-MHC-peptide complexes with a low affinity, it is possible that they would bind allo-MHC-peptide complexes with a high affinity, suggesting that a high affinity cross-reaction is responsible for the allorecognition observed. Furthermore, the high density of ligands expressed by donor APCs can further facilitate the activation of alloreactive T cells. This hypothesis is termed the 'high determinant density' hypothesis and was originally proposed by Bevan in 1984 (Fig. 1b) [27].

In 1989, Schneck et al. [28] developed a peptide which mimicked a specific region of the MHC class I molecule H-2K^b. In the presence of this peptide, the cytotoxic activity of H-2K^b-specific CD8$^+$ T cells against H-2K^b target cells was inhibited, demonstrating the importance of the TCR:MHC interaction in this alloresponse. Subsequently, and in contrast to the results of Wang et al. [22], Smith et al. demonstrated in a mouse setting that removal of peptides bound to MHC molecules through the use of acid washing did not perturb the ability of T cells to bind and react to allo-MHC molecules [29]. Lombardi et al. [30] proceeded to present corresponding findings in a human T cell setting. In this study, a site-directed mutagenesis approach was employed to generate genetically altered HLA-DR molecules which were transfected into murine DAP.3 cells. Using these artificial APCs, it was demonstrated that specific mutagenesis of TCR contact regions in the MHC molecule resulted in inhibition of T cell binding and subsequent effector responses. As such, it was evident that specific sites of the allo-MHC molecule were critical for direct allorecognition to occur. These findings were later confirmed by Villadangos et al. who employed a similar approach whilst mutating HLA-B27 [31].

Conundrum of Allorecognition

T cell progenitors must undergo a stepwise 'education' process in the thymus to develop into mature T cells. Thymocytes expressing a TCR capable of recognising peptides presented in the context of self-MHC molecules are 'positively selected', whilst thymocytes that recognise self-MHC-peptide complexes with a high affinity are 'negatively selected'. As such,

the mature T cells which results from this process are able to recognise self-MHC-peptide complexes with a low affinity.

The existence of this process reveals a conundrum: why do self-restricted T cells [32] recognise allo-MHC-peptide complexes? Studies have demonstrated that cross-reactivity between self and allogeneic MHC-peptide complexes is the key for this mode of allorecognition. In other words, T cells specific for peptide 'x' presented by self-MHC 'A' are also able to recognise peptide 'y' presented by allo-MHC 'B' [33]. Studies supporting this have demonstrated that LFA-3^+ [34] and $CD45RO^+$ [35] memory T cells primed against peptide 'x' presented by self-MHC 'A' also respond to allo-MHC-peptide complexes (peptide 'y' presented by allo-MHC 'B'). Furthermore, these cross-reacting memory T cells comprise a significant proportion of the total T cells which respond in a direct manner. This cross-reactivity concept was further accentuated by Lombardi et al. in 1989 [36]. In this study, human alloreactive T cell clones which were specific for HLA-DR1 were co-cultured with autologous APCs presenting *Candida albicans*-derived antigens in the context of HLA-DR4/HLA-DR13. Half of the alloreactive T cell clones analysed responded in these co-cultures, suggesting that cells which were capable of recognising the allo-MHC molecule HLA-DR1 had previously been activated by APCs presenting self-MHC-peptide complexes.

Structural Importance of TCRs and MHCs

The hypotheses and accompanying functional studies described have suggested that the attention of T cells with direct allospecificity is either for the allopeptides or the allo-MHC molecules. However, further insight into the molecular mechanisms of these interactions was provided by structural studies which employed x-ray crystallography. In 1987, Bjorkman et al. first published the structure of HLA-A2 [37]. Subsequent studies performed with HLA-A2 [38] and HLA-B27 [39] suggested specific residues which were important for allorecognition. However, significant progress in this field was not made until 1996 when co-crystals containing a TCR interacting with an MHC-peptide complex were generated [40, 41]. These studies confirmed for the first time that TCRs bind MHC-peptide complexes in a diagonal orientation, demonstrating that both the MHC and the peptide are recognised. Specifically, the variable Vα domain of the TCR positions above the N-terminal half of the peptide and the Vβ domain locates above the C-terminal half of the peptide [42, 43]. This places an emphasis on the complementarity-determining region (CDR)3 loops of the TCR [44], the most variable region of the TCR, for recognising the peptide whilst the CDR1 and CDR2 loops primarily interact with the MHC.

These studies accentuate the plasticity with which a TCR is able to cross-react and engage a variety of different MHC-peptide complexes [45, 46]. Indeed, this TCR degeneracy (ability of a single TCR to engage multiple MHC-peptide complexes) has fundamental ramifications in that it allows a finite number of T cells to recognise a potentially infinite number of MHC-peptide complexes [46, 47]. Although various theories exist to explain this degeneracy [48], it is clear that the ability of a TCR to change conformation upon engagement of a cognate MHC-peptide complex is paramount [42].

In 2007, Colf et al. [49] demonstrated that whilst a single TCR could cross-react to recognise self-MHC and allo-MHC molecules, different TCR conformations were required to accomplish this. The structure of the mouse-based TCR '2C' was compared when engaged with the self-MHC-peptide complex $H2K^b$-dEV8 and the allo-MHC-peptide complex $H2L^d$-QL9 [50]. Genetic manipulation of the CDR3α to yield a high-affinity variant of the 2C TCR did not influence the orientation with which the allo-MHC-peptide complex $H2L^d$-QL9 was bound. As such, interrupting the CDR3α-peptide interaction had little effect on the binding of the TCR to the allo-MHC-peptide complex, suggesting an MHC-centric model may be prevalent in this setting [50].

Conversely, structural data has also suggested a crucial role for allopeptides in driving direct allorecognition. Studies by Reiser et al. [44, 51, 52] conducted using mouse-based TCRs have detailed key conformational changes which occur in the CDR3 loops of a TCR upon binding of an allogeneic MHC class I molecule. Here, the CDR3α loop of the TCR 'BM3.3' was found to adopt different conformations depending on the peptide presented: presentation of the 'pBM1' peptide caused the CDR3α loop to fold away from the peptide binding groove [52] but when the 'VSV8' peptide was presented, the CDR3α loop pointed towards the amino-terminal end of the peptide [51]. Furthermore, given the fact that relatively minor changes were observed in the CDR1 and CDR2 loops, these results suggest that the allopeptide is responsible for driving direct allorecognition [48, 53]. Studies conducted with human TCRs interacting with HLA-B molecules have also yielded results in favour of a peptide-dependent direct allorecognition model. It has been suggested that MHC molecular mimicry is the basis for the cross-reactivity observed between self-HLA-B*0801 and allo-HLA-B*3501 [54] as well as self-HLA-B*0801 and allo-HLA-B*4402/4405 [55]. In the latter study, the nature with which the TCR 'LC13' interacted with self-HLA-B*0801 and allo-HLA-B*4405 was remarkably similar; a comparable number of van der Waals interactions, hydrogen bonds and salt-bridges were formed in each case. Additionally, despite the fact that drastically different peptides were presented, very similar contacts were made by the CDR loops interacting with the self- and allo-HLA-B molecules.

Overall, the aforementioned high determinant density and multiple binary complex models provide two explanations for why T cells with direct allospecificity exist with a high-precursor frequency. With functional and structural data supporting both hypotheses, it is likely that in vivo, the high frequency of direct allorecognition can be attributed to a combination of these theories.

Consequences of Direct Allorecognition

Allorecognition typically leads to an effector response in which $CD8^+$ T cells with direct allospecificity actively kill donor-derived target cells [56], leading to allograft dysfunction and failure. Various studies have investigated how recipient graft-specific $CD8^+$ and $CD4^+$ T cells contribute to acute and chronic transplant rejection [33, 57–60]. For example, in 2000, Pietra et al. [61] investigated the contribution of $CD4^+$ T cells in acute graft rejection through the use of severe combined immunodeficiency (SCID) and recombination-activating gene (RAG)-1 deficient mice which lack functional T and B cells. C57BL/6 heart allografts, which survived indefinitely in SCID mice, were acutely rejected (mean survival time (MST) of 12 days) when BALB/c $CD4^+$ T cells were adoptively transferred on the day of transplant. Conversely, heart allografts which lacked donor C57BL/6 MHC class II molecules (C2D donor mice) were not rejected (7/8 allografts survived >60 days), demonstrating that $CD4^+$ T cells directly specific for donor MHC class II molecules were necessary for acute allograft rejection.

More recently, Brown et al. [62] further demonstrated the contribution of recipient $CD4^+$ T cells to allograft rejection using a fully mismatched kidney transplant model in which donor APCs were specifically depleted. In this study, one native kidney of recipient (FVB) mice was replaced with an allogeneic (C57BL/6 × CBA F1) kidney. In the absence of treatment, transplanted kidneys were rejected acutely in 40 % of cases. However, in recipients which were treated with an immunotoxin-conjugated antibody specific for donor MHC class II ($I\text{-}A^k$) to depleted donor APCs, kidney allografts were completely protected: histological analysis showed no evidence of rejection and upon removal of the second native kidney, the function of the transplanted kidney (blood urea nitrogen score) was found to be intact.

Analysis of blood samples acquired from stable renal transplant recipients has revealed that recipient $CD4^+$ T cells with direct allospecificity become hyporesponsive towards alloantigens and are not deleted [63]. This work was further extended by demonstrating that that human $CD4^+$ T cells co-cultured with MHC class II-expressing thyroid follicular cells (TFC) [64] or epithelial cells [65] do not proliferate or produce cytokines in the absence of co-stimulation and are hyporesponsive upon subsequent challenge with EBV-transformed lymphoblastoid B cell lines (B-LCL) [66]. Together, these results suggest that in the absence of donor-derived professional APCs, recipient $CD4^+$ T cells engage MHC class II molecules presented by transplanted tissue parenchymal cells which lack co-stimulatory molecules. As a result, these T cells become anergic [65] or polarised towards a Th2 phenotype [64], suggesting that prolonged alloimmune responses depend on an alternative mode of allorecognition.

Sustaining a Long-Term Direct Alloimmune Response

During a transplant procedure, donor APCs are transferred but these cells are killed or die over time [56]. As such, it has historically been believed that the direct pathway of allorecognition predominates during acute graft rejection. Furthermore, as previously discussed, in the absence of donor APCs, T cells with direct allospecificity recognise allo-MHC presented on the allograft parenchyma which leads to anergy induction [66].

As previously discussed, Lechler and Batchelor [13, 14] demonstrated that prolonged rat kidney allograft survival could be achieved by 'parking' the allograft in an intermediate recipient to deplete passenger leucocytes. However, the fact that these allografts were eventually rejected led to the proposal of the indirect pathway of allorecognition whereby recipient-derived APCs consistently sample and present donor antigens provided by the allograft. As such, the indirect pathway has been believed to be the main mode of chronic rejection [67].

As antigens acquired from exogenous origins are naturally presented in the context of MHC class II, it is primarily $CD4^+$ T cells which recognise alloantigens in an indirect manner, not $CD8^+$ T cells which are responsible for eliciting cytotoxicity. Furthermore, efficient $CD8^+$ T cell activation requires help from $CD4^+$ T cells [60]. As such, it is reasonable to conclude that a link between the direct and indirect pathways of allorecognition exists and that $CD4^+$ T cells with indirect allospecificity facilitate the activation of $CD8^+$ T cells with direct allospecificity [59, 68]. Theoretically, for this to occur, two separate APCs should be present: a recipient APC presenting allopeptides indirectly to the $CD4^+$ T cell and a donor APC presenting antigen directly to the $CD8^+$ T cell. However, from a practical viewpoint, it is highly unlikely that these two APC:T cell interactions naturally occur in such close proximity, leading to a conundrum termed the 'four-cell conundrum'. This was resolved by the discovery of APCs which present intact donor MHC-peptide complexes in a direct manner and allopeptides in an indirect manner [69–71], the basis of a more recently described third pathway of allorecognition: the semi-direct pathway [70].

In a similar manner to the indirect pathway, the semi-direct pathway relies on recipient-derived APCs which infiltrate the allograft following engraftment. However, in addition to presenting allopeptides indirectly, these cells acquire intact donor MHC-peptide complexes from donor-derived cells/tissues, a phenomenon termed 'cross-dressing', thus present allogeneic MHC-peptide complexes in a direct manner [71]. Indeed, DCs presenting both donor MHC class I-peptide complexes (direct presentation) and allopeptides in the context of self-MHC (indirect presentation) have been observed following skin [72••], kidney [73] and heart [74, 75••] transplantation. Through this pathway, direct alloimmune responses can continue long after donor-derived APCs have died, but the extent to which this

pathway contributes to allograft rejection is not yet known. Recently, we have shown that after removal of the direct pathway and in the absence of cross-presentation, acquired allo-MHC-peptide complexes, on recipient DCs, can drive allograft rejection throughout the life-span of the transplant [72].

Targeting T cells with Direct Allospecificity

Over the past few decades, advances in surgical techniques and the development of modern immunosuppressive regimens have enabled transplantation of cells/organs to become a viable treatment option for a plethora of different conditions. Although the mechanisms by which these immunosuppressive drugs function are not completely understood, it is believed their benefits stem through the suppression of T cells with direct allospecificity [76]. However, drug-based immunosuppression is inherently non-specific and associated with undesirable side-effects, leaving recipients under consistent nephrotoxic insult with an increased susceptibility to acquiring infections and developing cancer [76]. As such, various strategies are currently under investigation to reduce the requirement for these drugs.

Following transplantation, recipient T cells with direct allospecificity are initially activated by 'passenger leukocytes'. Given the deleterious consequences of these cells, strategies have been explored to investigate whether depletion of these cells from allografts can reduce the intensity of an initial immune insult. Brown et al. [62] recently achieved this through the use of a donor MHC class II-specific immunotoxin-conjugated antibody. As described above, these authors observed indefinite kidney allograft survival and function in mice which received this treatment. Additionally, Stone et al. [77•] have demonstrated that the proportion of passenger leukocytes in lung allografts can be significantly reduced through the use of ex vivo lung perfusion (EVLP). These authors employed a model whereby donor pig lungs are explanted, perfused ex vivo and then transplanted into recipient pigs. EVLP, which did not severely alter the viability of the graft, reduced both donor leukocyte egression and recipient T cell infiltration post-transplantation, suggesting a potential clinical benefit of passenger leukocyte removal prior to allograft implantation.

Luo et al. have explored the possibility of inducing donor-specific transplant tolerance by infusing ethylene carbodiimide (ECDI)-fixed donor-derived APCs before and after a transplant procedure [78]. Fuelled by similar approaches which were applied to treat multiple sclerosis [79] and diabetes [80] in mice, the authors of this study demonstrated that indefinite survival (>100 days) of fully mismatched islets could be achieved by infusing recipient mice intravenously with 100 million donor-derived ECDI-treated splenocytes before and after the islet transplant procedure. Furthermore, it was demonstrated that $CD4^+CD25^+$ Tregs had a crucial role in tolerance induction. These authors have also demonstrated that similar levels of tolerance can be achieved through the use of biodegradable particles (poly (lactide-co-glycolide); PLG) which present donor antigens (PLG-dAg) as a substitute for the aforementioned ECDI-treated splenocytes [81].

As suggested, Tregs play a fundamental role in inducing tolerance in vivo. Allograft survival is significantly prolonged by increasing the proportion of Tregs in recipient mice, either by promoting endogenous Treg expansion [82] or adoptively transferring ex vivo-expanded Tregs [83, 84]. Similarly, observational studies performed on human samples have noted a correlation between the proportion of Tregs and allograft survival [85, 86]. These studies paved the way for phase I/II clinical trials which are currently investigating the safety and efficacy of polyclonal Treg therapy in kidney (The ONE Study), liver (ThRIL) and bone marrow transplant recipients [87]. Furthermore, we [88•, 89] and others [90] have demonstrated that Tregs with direct allospecificity are superior to polyclonal Tregs at prolonging allograft survival in vivo. In these studies, human Tregs with direct allospecificity were preferentially expanded using allogeneic DCs [88•] or B cells [89, 90]. Using human skin xenograft transplant models, direct allospecific Tregs were shown to inhibit direct alloimmune-mediated skin injury significantly more effectively than polyclonal Tregs. However, we have also observed in a mouse setting that Tregs with direct allospecificity alone are insufficient to prolong the survival of heart allografts [91]. To achieve this, Tregs required both direct and indirect allospecificity, suggesting that it is necessary to block both direct and indirect allospecific Teffs in order to reduce allograft damage in this setting. Given the superior efficacy of Tregs with direct allospecificity, compared to polyclonal Tregs, the safety and efficacy of these cells is currently being assessed clinically in kidney (DART as part of The ONE Study: NCT02244801) and liver (deLTa: NCT02188719 and NCT01624077) transplant recipients.

Conclusions

The mechanisms of direct allorecognition have puzzled immunologists for decades. Why self-restricted T cells recognise allo-MHC-peptide complexes and with a high-precursor frequency remain a mystery. Theories proposed to offer an explanation for these conundrums attribute the phenomenon of direct allorecognition to either the presence of allopeptides or allo-MHC molecules. Both theories are supported by functional and structural data, suggesting that in vivo, both allopeptides and allo-MHC molecules are responsible for driving direct allorecognition. For many decades, the direct pathway of allorecognition was believed to be solely responsible for early alloimmune-mediated rejection. However, the

more recent discovery of the semi-direct pathway by our group has demonstrated how rejection mediated by T cells with direct allospecificity can be sustained long-term. As such, various strategies are currently being explored as potential means of limiting direct allorecognition and inducing tolerance.

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Compliance with Ethical Standards

Conflict of Interest Dominic Boardman, Giovanna Lombardi, Jacinta Jacob, Lesley Smyth and Robert Lechler declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Expression of a Chimeric Antigen Receptor Specific for Donor HLA Class I Enhances the Potency of Human Regulatory T Cells in Preventing Human Skin Transplant Rejection

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Regulatory T cell (Treg) therapy using recipient-derived Tregs expanded *ex vivo* is currently being investigated clinically by us and others as a means of reducing allograft rejection following organ transplantation. Data from animal models has demonstrated that adoptive transfer of allospecific Tregs offers greater protection from graft rejection compared to polyclonal Tregs. Chimeric antigen receptors (CAR) are clinically translatable synthetic fusion proteins that can redirect the specificity of T cells toward designated antigens. We used CAR technology to redirect human polyclonal Tregs toward donor-MHC class I molecules, which are ubiquitously expressed in allografts. Two novel HLA-A2-specific CARs were engineered: one comprising a CD28-CD3ζ signaling domain (CAR) and one lacking an intracellular signaling domain (ΔCAR). CAR Tregs were specifically activated and significantly more suppressive than polyclonal or ΔCAR Tregs in the presence of HLA-A2, without eliciting cytotoxic activity. Furthermore, CAR and ΔCAR Tregs preferentially transmigrated across HLA-A2-expressing endothelial cell monolayers. In a human skin xenograft transplant model, adoptive transfer of CAR Tregs alleviated the alloimmune-mediated skin injury caused by transferring allogeneic peripheral blood mononuclear cells more effectively than polyclonal Tregs. Our results demonstrated that the use of CAR technology is a clinically applicable refinement of Treg therapy for organ transplantation.

Abbreviations: ANOVA, analysis of variance; APC, antigen-presenting cell; B-LCL, B-lymphoblastoid cell line; BRG, BALB/c Rag2$^{-/-}\gamma_c^{-/-}$; CAR, chimeric antigen receptor; CLA, cutaneous lymphocyte antigen; eGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-assisted cell sorting; FOXP3, factor forkhead-box protein 3; GMP, good manufacturing practice; GvHD, graft-versus-host disease; HUVEC, human umbilical cord endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCT, optimal cutting temperature; PBMC, peripheral blood mononuclear cell; RAG, recombination activating gene; scFv, single-chain variable fragment; SD, standard deviation; SEM, standard error of the mean; Teff, effector T cell; Treg, regulatory T cells

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Introduction

The long-term benefits of organ transplantation are hindered by graft rejection (1–5), a detrimental process driven by alloreactive T cells that recognize donor MHC antigens via the direct, indirect, and semidirect pathways of allorecognition (3,6). Current immunosuppressive regimens target the direct pathway and reduce acute allograft rejection (7) but are associated with severe

side-effects (8) and do not effectively prevent chronic rejection (2,9); thus, the half-life of allografts remains limited to 10–15 years (10,11).

Thymus-derived regulatory T cells (tTregs) are immunosuppressive T cells with a fundamental role in the maintenance of tolerance *in vivo* (12–15). These cells are characterized in humans as $CD4^+CD25^{hi}CD127^{lo}$ and constitutively express the transcription factor forkhead-box protein 3 (FOXP3) (7,12,16). In rodent models, Tregs significantly prolong the survival of skin (17–19) and heart (4) allografts and in humans, correlations between the proportion of Tregs within allografts and graft survival have been observed (20–22). With the safety of Treg therapy having been demonstrated in the contexts of graft-versus-host disease (GvHD) (23–26) and type I diabetes (27,28), we are conducting Phase I/II clinical trials to investigate the therapeutic potential of adoptive polyclonal Treg therapy to promote tolerance in kidney (The ONE Study: NCT02129881) and liver (ThRIL: NCT02166177) transplant recipients (29,30).

However, we (4,17,19,31) and others (32,33) have demonstrated in animal models that allospecific Tregs are superior to polyclonal Tregs at reducing graft rejection, upon adoptive transfer. Murine Tregs expanded with allogeneic antigen-presenting cells (APC) (direct allospecificity) and/or transduced to express an allospecific TCR (indirect allospecificity) prolonged graft survival significantly more effectively than polyclonal Tregs. These findings were confirmed using human Tregs expanded with allogeneic dendritic cells (19) and B cells (31,34) in human skin xenograft transplant models. As such, clinical trials are assessing the safety and efficacy of Tregs with direct allospecificity in kidney (DART: NCT02188719) and liver (deLTa: NCT02188719 and NCT01624077) transplant recipients. Here, we investigated whether allospecificity may be conferred onto Tregs using chimeric antigen receptors (CAR) (35).

CARs are synthetic fusion proteins capable of redirecting the specificity of T cells toward desired antigens. Classical CARs comprise an extracellular antigen-targeting moiety that binds a specific antigen in an MHC-independent manner and a series of customized intracellular TCR and costimulatory signaling domains. As such, CARs translate the binding of specific antigens into the activation of desired signaling cascades (36). CARs are primarily used clinically to generate tumor antigen–specific T cells (37–43). However, preclinical studies have shown the efficacy of CAR-modified Tregs for the treatment of colitis (16,44,45) and multiple sclerosis (46). The functionality of CARs has also been demonstrated in human Tregs (47,48), most recently as a means of protecting against xeno-GvHD (49). However, to date, no one has demonstrated the capacity of CAR Tregs to protect from solid transplant rejection.

In this study, we applied CAR technology to generate allospecific Tregs with the aim of promoting organ transplant tolerance. We redirected human $CD4^+CD25^+CD127^{lo}FOXP3^+$ Tregs toward an MHC class I molecule (HLA-A2), an alloantigen that is ubiquitously expressed in an allograft, with the hypothesis that they would be more potent than polyclonal Tregs at protecting from graft rejection, upon adoptive transfer.

Materials and Methods

CAR generation

A previously described (50) HLA-A2-specific single-chain variable fragment (scFv) sequence (3PB2 V_H and DPK1 V_L) was validated by immunoprecipitation (data not shown) and cloned upstream of (i) a 9E10 cMyc epitope; (ii) a human CD28 hinge/transmembrane domain; (iii) a human CD28-CD3ζ signaling domain; and (iv) an enhanced green fluorescent protein (eGFP) open reading frame in a second-generation pLNT/SFFV lentiviral plasmid (provided by Prof. Adrian Thrasher; UCL, London, UK). A truncated CAR (ΔCAR) was generated by removing the CD28-CD3ζ signaling domain from the full-length CAR-eGFP fusion. All plasmids were verified by sequencing (Beckman Coulter Genomics, Takeley, Essex, UK).

Human $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ Teff isolation and culture

Anonymized healthy donor peripheral blood was obtained from the National Blood Service (NHS Blood and Transplantation, Tooting, London, UK) with informed consent and ethical approval (Institutional Review Board of Guy's Hospital; reference 09/H0707/86). $CD4^+$ T cells were enriched using RosetteSep™ (StemCell Technologies, Waterbeach, Cambridgeshire, UK), after which $CD4^+CD25^-$ Tregs and $CD4^+CD25^-$ Teffs were separated using CD25 MicroBeads II (Miltenyi Biotec, Bisley, Surrey, UK).

Tregs/effector T cells (Teffs) were activated with anti-CD3/CD28 beads (1:1 bead:cell ratio; Thermo Fisher Scientific, Paisley, Renfrewshire, UK) and cultured in X-vivo™ 15 (Lonza, Slough, Berkshire, UK) supplemented with 5% human AB serum (BioSera, Uckfield East, Sussex, UK). Treg were cultured with 100 nM rapamycin (LC-Laboratories, Woburn, MA) and 1000 U/mL recombinant IL-2 (Proleukin-Novartis, Camberley, Surrey, UK), whereas Teffs were cultured with 100 U/mL IL-2 only.

Flow cytometry and cell sorting

Cells were stained in phosphate-buffered saline (PBS) supplemented with 1% heat-inactivated fetal calf serum (FCS) and 5 mM EDTA (all from Thermo Fisher Scientific) using fluorescently conjugated antibodies specific for HLA-A2 (BB7.2), CD4 (OKT4), FOXP3 (PCH101), CD39 (eBioA1), CD69 (H1.2F3) (all from eBioscience, Hatfield, Hertfordshire, UK), CD25 (4E3 or 2A3), CTLA-4 (BNI3) (all from BD Biosciences, Oxford, Oxfordshire, UK) CD127 (A019D5), CCR4 (L291H4), CCR9 (L053E8), CCR10 (5688-5), CD62L (DREG-56), integrin β7 (FIB504), CLA (HECA-452), HLA-DR (all from BioLegend, London, UK), HLA-A2 (REA142) (Miltenyi-Biotec), and HLA class I (Tu149) (Thermo Fisher Scientific). Cells were stained with PE-conjugated HLA-A*0201/CINGVCWTV and HLA-B*0702/DPRRRSRNL dextramers (Immudex, Copenhagen, Denmark) provided by Dr. Marc Martinez-Llordella; KCL, London, UK. Dead cells were excluded using live/dead near-IR staining (Thermo Fisher Scientific). Intracellular staining was performed using the eBioscience Fix/Perm kit. Cells were acquired and sorted using an LSRFortessa II and FACSAria II (BD), respectively. Data were analyzed using FlowJo 7 or 10 (Tree Star, Ashland, OR).

Immortalized cell line culture

HEK293T, MCF-7, and T-47D epithelial cells were grown in DMEM-based media. B-Lymphoblastoid cell lines (B-LCL) and K562s (donated by Dr. Marc Martinez-Llordella) were grown in RPMI-based media. All culture media were supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Thermo Fisher Scientific). HLA-transfected K562 cultures were supplemented with 5 μg/mL neomycin (Thermo Fisher Scientific). All cells were grown at 37°C in the presence of 5% (vol/vol) CO_2.

Lentivirus production and Treg/Teff transduction

HEK293T cells were cotransfected with pLNT/SFFV-CAR-eGFP or pLNT/SFFV-ΔCAR-eGFP (Figure 1A), pΔ8.91 and pCMV-VSV-G plasmids at a mass ratio of 4:3:1 using polyethylenimine (3:1 PEI:DNA wt/wt; Sigma-Aldrich, Gillingham, Dorset, UK). Viral supernatant was harvested 48–56 h posttransfection and lentiviral particles were concentrated using PEG-it™ (System Biosciences, Bar Hill, Cambridgeshire, UK). Tregs/Teffs were transduced in RetroNectin®-treated plates (50 μg/μL; Takara Bio Inc., Saint-Germain-en-Laye, Yvelines, France) 3 days postisolation using four-fold concentrated viral supernatant. eGFP$^+$ cells were purified by fluorescence-assisted cell sorting (FACS) 7 days posttransduction.

T cell/epithelial cell coculture

Teffs/Tregs were cocultured overnight with confluent MCF-7 or T-47D cell monolayers. Culture supernatants were collected to measure IL-2, interferon (IFN)γ, and IL-10 production by cytokine-specific enzyme-linked immunosorbent assays (ELISA) (eBioscience). Monolayers were washed and the viability of the monolayer cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Absorbance was measured at 560 nm. Results are shown as percent viability relative to monolayers cultured alone.

Suppression assays

Tregs were cocultured with autologous CD4$^+$CD25$^-$ Teff responders that were labeled with CellTrace Violet (CTV; Thermo Fisher Scientific) and activated with anti-CD3/CD28 beads (1:40 bead:cell ratio) or irradiated (12 000 cGy) allogeneic B-LCLs (3:1 T:B-LCL ratio); DBB (HLA-A2$^+$DR7$^+$), MOU (HLA-A2$^-$DR7$^+$), SPO (HLA-A2$^+$DR11$^+$), and BM21 (HLA-A2$^-$DR11$^+$). Teff CTV dilution was measured after 5 days using flow cytometry. Results are shown as percent suppression (inverse of percent Teff proliferation) relative to Teffs cultured alone.

In vitro *flow chamber assay*

Primary human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion using ethically approved protocols (East London & The City Local Research Ethics Committee reference 05/Q0603/34 ELCHA). HUVECs were stimulated with 15 ng/mL IFNγ (R&D Systems, Abingdon, Oxfordshire, UK) for 72 h prior to experimentation and seeded in μ-Slides VI 0.4 (Ibidi, Planegg/Martinsried, Germany) coated with 0.5% bovine gelatin. Tregs were suspended at 1×10^6 cells/mL in PBS (with Ca^{2+} and Mg^{2+}) and flowed across HUVEC layers using a shear stress of 1 dyn/cm^2. The number of Tregs that migrated in 10-s frames was assessed.

Animals

BALB/c recombination activating gene (RAG)2$^{-/-}\gamma_c^{-/-}$ (BRG) mice were maintained under sterile conditions (Biological Services Unit, New Hunt's House, King's College London). All procedures were performed in accordance with all legal, ethical, and institutional requirements (PPL70/7302).

Human skin xenograft transplant model

Human skin was obtained from routine surgical procedures with informed consent and ethical approval (Guy's and St. Thomas' NHS Foundation Trust and King's College London; reference 06/Q0704/18). Donor HLA-A2 expression was determined by flow cytometry, staining skin-derived cells obtained by collagenase digestion (100 μg/mL; Sigma-Aldrich). Split-thickness skin grafts (1.5 cm^2) were transplanted onto 10–11-week-old recipient BRG mice as previously described (19) and mice were administered 100 μg anti-mouse Gr-1 (BioXCell, Upper Heyford, Oxfordshire, UK) intraperitoneally every 3–4 days. After 5–6 weeks (Figure S4), mice were injected intravenously with 5×10^6 peripheral blood mononuclear cells (PBMCs) ± 1×10^6 Tregs. Skin grafts were harvested for histological analysis 5 weeks following PBMCs/Tregs transfer.

Histological analysis of human skin

Skin grafts were frozen in optimal cutting temperature (OCT) (Thermo Fisher Scientific). Eight- or 16-μm-thick sections were fixed in 4% paraformaldehyde, blocked with a mixture of 10% donkey serum, 0.1% fish skin gelatin, 0.1% Triton X-100, and 0.5% Tween-20 (all from Sigma-Aldrich) in PBS and stained with the following antibodies: anti-human CD3 (polyclonal rabbit; DAKO, Stockport, Cheshire, UK), anti-FOXP3 (236A/E7; Abcam, Milton, Cambridgeshire, UK), anti-CD45 (HI30, eBioscience), anti-involucrin (CY5; Sigma-Aldrich), anti-human CD31, and Ki67 (both polyclonal rabbit; Abcam). Sections were stained with secondary donkey anti-mouse Alexa Fluor®555 and anti-rabbit Alexa Fluor®488 or Alexa Fluor®647 antibodies with 4′,6-diamidino-2-phenylindole (DAPI) (all from Thermo Fisher Scientific) and mounted with Fluorescence Mounting Medium (DAKO). Maximum intensity projection images consisting of 10 z-stacks (1.1 μm apart) were acquired at ×20 magnification using a C2+ point scanning confocal microscope (Nikon, Kingston Upon Thames, Surrey, UK) and analyzed/quantified with NIS Elements and FIJI imaging software (51).

Statistical analysis

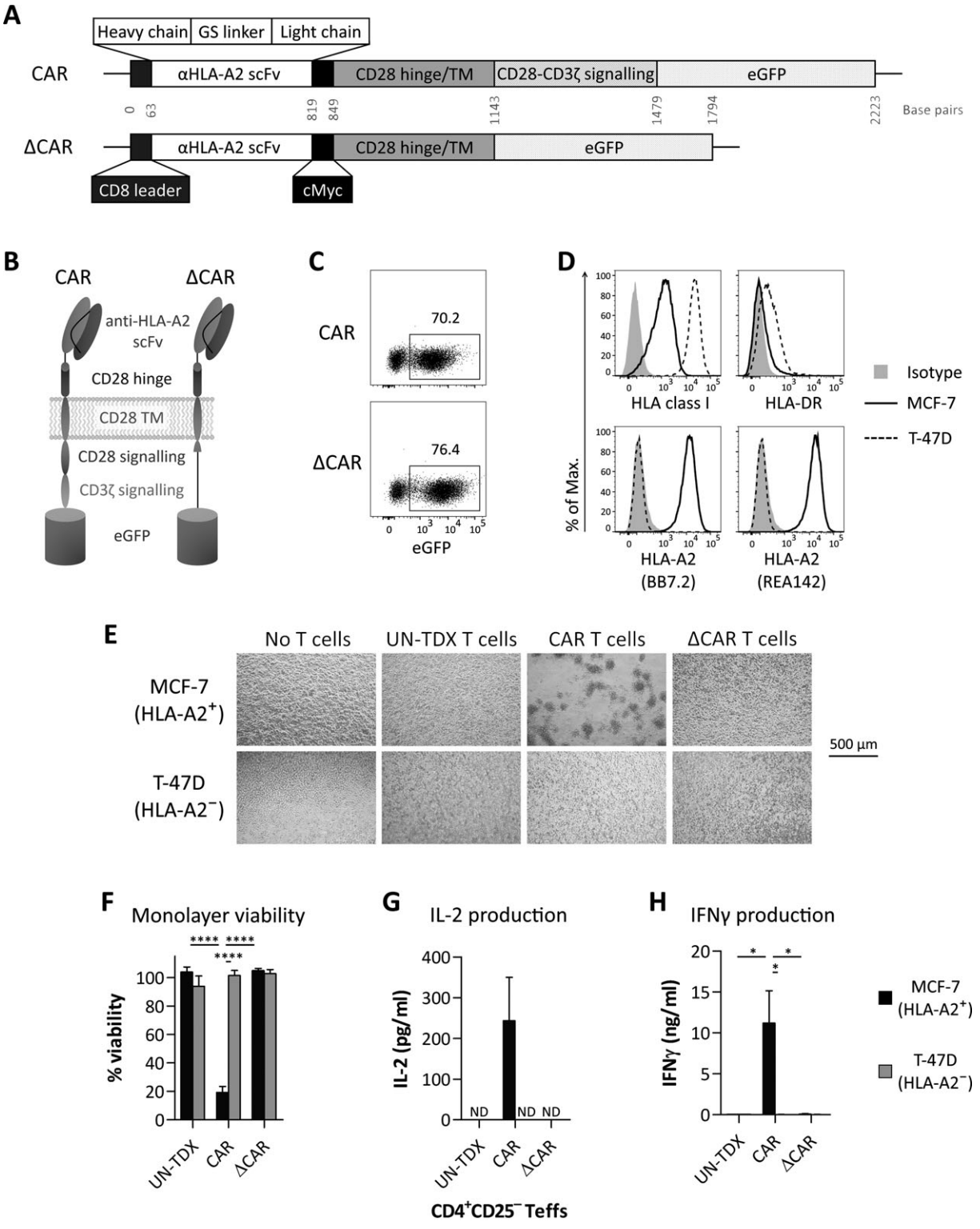
Data shown are mean ± SEM or mean ± SD. Statistical significance was determined using two-tailed paired Student's t-tests or analysis of variance (ANOVA) with the Tukey multiple comparison post-hoc test. $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$.

Results

Generation and validation of a novel HLA-A2-specific CAR

A HLA-A2-specific CAR incorporating a human CD28-CD3ζ signaling domain was generated using a patient-derived HLA-A2-specific scFv sequence (50) (Figure 1A and B). A second-generation CAR was selected based on the superior function of these CARs relative to first-generation CARs (52–55) and the importance of CD28 signaling for Treg activation (56). To investigate the importance of this signaling domain, a HLA-A2-specific CAR lacking a CD28-CD3ζ signaling domain (ΔCAR) was also generated (Figure 1A and B). This control was selected, as opposed to a CAR containing a scrambled ectodomain, to ascertain whether the ability of the Tregs to engage HLA-A2 was sufficient to elicit protection *in vivo* or whether Treg activation via the CAR was also necessary.

To confirm the functionality and HLA-A2-specificity of these CARs, polyclonal CD4$^+$CD25$^-$ effector T cells (Teffs) were transduced with VSV-G-pseudotyped lentiviral particles containing the CAR or the ΔCAR constructs



with an efficiency of 40–80% (54.3–62.8 ± 11.9%; Figure 1C). eGFP$^+$ Teffs were purified by cell sorting and cocultured overnight with confluent monolayers of MCF-7 and T-47D cells, breast cancer epithelial cells that differentially expressed HLA-A2 (Figure 1D (57)). CAR Teffs specifically destroyed HLA-A2$^+$ monolayers but left HLA-A2$^-$ monolayers intact (Figure 1E). Compared to epithelial cells cultured alone, CAR Teffs killed

Figure 1: CD4$^+$CD25$^-$ T cells expressing the full-length CAR were activated in the presence of HLA-A2$^+$ cells. (A, B) Schematic diagrams detailing the components of the CAR genes. A CAR–eGFP fusion protein was generated consisting of a HLA-A2-specific scFv (white), a CD28 hinge/transmembrane (TM) domain (gray), a CD28/CD3ζ signaling domain (light gray), and an eGFP reporter gene (gray dots). A control CAR (ΔCAR) comprising the same elements but no signaling domain was also generated. (C) Transduction efficiency of CD4$^+$CD25$^-$ Teffs, prior to cell sorting, as determined by flow cytometry analyzing eGFP expression. Data are representative of four individual experiments. (D) Flow cytometry histogram plots showing the expression of HLA class I, HLA-DR and HLA-A2 (BB7.2 or REA142 clone) by MCF-7 (solid line) and T-47D (dotted line), compared to an isotype control (solid gray). (E) Microscopic images of MCF-7 and T-47D monolayers following overnight coculture with untransduced (UN-TDX), CAR, or ΔCAR T cells. Representative data of four individual experiments. (F) Quantification of MCF-7 (HLA-A2$^+$, black) and T-47D (HLA-A2$^-$, gray) viability following overnight coculture with untransduced, CAR or ΔCAR T cells as measured by MTT assay. Percent viability is relative to monolayers cultured alone. IL-2 (G) and IFNγ (H) production by the T cells during coculture with cell monolayers, as measured by ELISA. Data shown are mean ± SEM pooled from four individual experiments and significance was determined by two-tailed paired Student's t-test where * = $p < 0.05$ and **** = $p < 0.0001$. ND = not detected. CAR, chimeric antigen receptor; ΔCAR, truncated chimeric antigen receptor; eGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; IFNγ, interferon γ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; scFv, single-chain variable fragment; SEM, standard error of the mean; Teffs, effector T cells; UN-TDX, untransduced.

80.8 ± 4.2% of the HLA-A2$^+$ cells but not the HLA-A2$^-$ cells (Figure 1F). Untransduced and ΔCAR Teffs exhibited no detectable level of cytotoxicity. High levels of IL-2 (Figure 1G) and IFNγ (Figure 1H) were also produced by the CAR Teffs during coculture with HLA-A2$^+$ cells. These results demonstrated that CAR expression by T cells mediated specific recognition of HLA-A2 antigens resulting in Teff activation, cytotoxicity, and cytokine production.

Human CD4$^+$CD25$^+$ Tregs maintained their phenotype and function following lentiviral CAR transduction

Having validated the CARs in Teffs, we next assessed whether lentiviral transduction influenced the phenotype and/or suppressive capacity of human Tregs. CD4$^+$CD25$^+$ Tregs were enriched using good manufacturing practice (GMP)-compatible protocols (58) from the peripheral blood of healthy HLA-A2 donors with a purity of approximately 90% (89.5 ± 4.4%, n = 16; Figure S1A). A high proportion of the cells were FOXP3$^+$ with a low expression of CD127 (Figure S1B). These cells were activated using anti-CD3/CD28 beads and expanded in the presence of 1000 U/mL recombinant human IL-2 and 100 nM rapamycin (29,58). Tregs were transduced with the CAR or ΔCAR construct 72 h postactivation (Figure 2A), with efficiencies of 30–80% (55.1–69.2 ± 20.3%, n = 15; Figure 2B). eGFP$^+$ Tregs were purified by flow sorting 7 days posttransduction (Figure 2B) and expanded for an additional 10 days (Figure 2A). All eGFP$^+$ Tregs were shown to have a detectable level of CAR expression on the cell surface (Figure 2B), which facilitated in the specific recognition of a HLA-A2-based dextramer but not an irrelevant HLA-B7-based dextramer (Figure 2C). Furthermore, engagement of HLA-A2 was shown to specifically activate CAR Tregs but not untransduced or ΔCAR Tregs (Figure 2D and Figure S2).

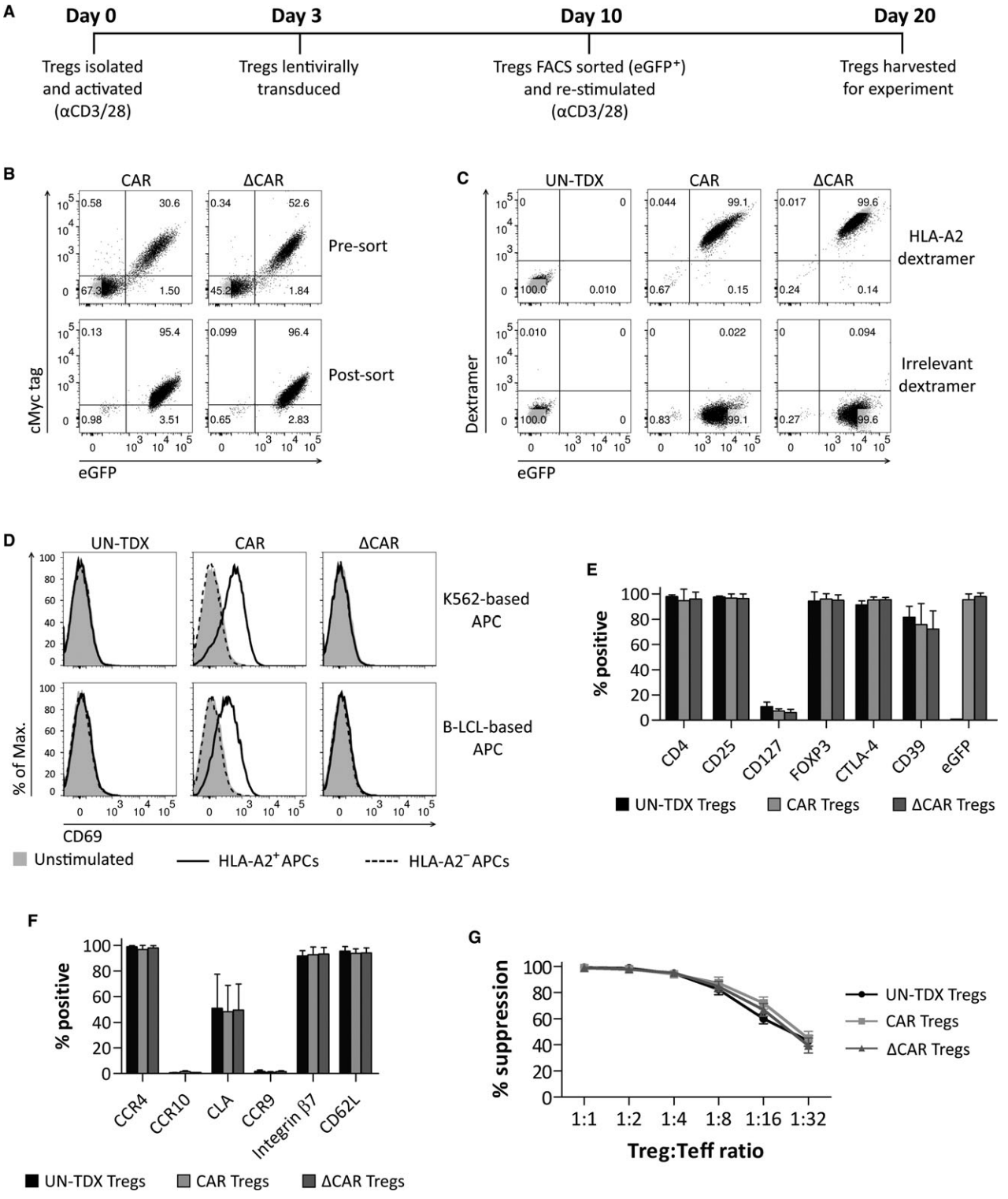
Following expansion, >95% (95.3–97.8 ± 4.7%) of the transduced Tregs remained eGFP$^+$. These cells exhibited a similar phenotype to untransduced Tregs (Figure 2E and Figure S3A); they remained CD4$^+$CD25$^+$CD127^{lo} with a high proportion expressing FOXP3 (94.2–95.8 ± 7.3%), CTLA-4 (91.3–95.3 ± 3.1%), and CD39 (72.4–81.7 ± 16.3%). Treg expression of specific homing receptors was also unaltered (Figure 2F and Figure S3B). The Tregs expressed the skin homing molecules CCR4 (96.7–98.8 ± 3.2%) and cutaneous lymphocyte antigen (CLA) (48.4–51.0 ± 26.4%) but few cells expressed CCR10 (0.6–1.5 ± 0.7%), as previously published (58). All cells expressed CD62L (93.7–95.6 ± 3.9%) and with regards to gut homing, a high proportion expressed integrin β7 (91.7–93.2 ± 5.9%) but few cells expressed CCR9 (1.1–1.7 ± 1.0%).

To assess the suppressive capacity of the Tregs, untransduced, CAR and ΔCAR Tregs were cocultured with CTV-labeled autologous CD4$^+$CD25$^-$ responder Teffs in the presence of anti-CD3/28 beads for 5 days. At a 1:32 Treg:Teff ratio, untransduced, CAR and ΔCAR Tregs inhibited Teff proliferation by 43.0 ± 4.1%, 44.8 ± 5.4%, and 39.4 ± 5.7%, respectively, proving the potency of these cells and indicating that the Treg function following transduction was unaltered.

Taken together, these results demonstrated that human Tregs isolated and expanded with clinically relevant protocols maintained their phenotype and function following transduction with VSV-G-pseudotyped lentiviral particles.

CAR-mediated alloantigen recognition by human Tregs enhanced their potency

To assess whether CAR Tregs were functionally superior to untransduced (polyclonal) Tregs in the presence of HLA-A2, Tregs were cocultured with CTV-labeled CD4$^+$CD25$^-$ responder Teffs, and allogeneic B-LCLs which differentially expressed HLA-A2 as APCs. The suppressive profile of the polyclonal Tregs cultured with HLA-A2$^+$ and HLA-A2 B-LCLs was identical (Figure 3A), whereas CAR Tregs inhibited Teff proliferation significantly more effectively (p-values 0.0082–1.1×10^{-5}) in



the presence of HLA-A2$^+$ B-LCLs, compared to HLA-A2 B-LCLs (Figure 3B). At a 1:32 and 1:64 Treg:Teff ratio, CAR Tregs inhibited Teff proliferation by 62.8 ± 5.2% and 38.1 ± 3.6%, respectively, in the presence of HLA-A2 but only 35.8 ± 3.5% and 16.9 ± 3.4%, respectively, in the absence of HLA-A2. ΔCAR Tregs were significantly more suppressive in the presence of HLA-A2 at Treg:Teff ratios of 1:2 ($p = 0.041$) and 1:32 ($p = 0.027$) (Figure 3C).

Figure 2: Human CD4$^+$CD25$^+$ Tregs maintained their phenotype and function following CAR lentiviral transduction and cell sorting. (A) Timeline showing production of human CAR Tregs used for experimentation. (B) Representative flow cytometry data showing the proportion of Tregs that were successfully transduced (eGFP expression) and the proportion of cells coexpressing the CAR/ΔCAR construct on the cell surface (c-Myc expression) immediately before and after eGFP$^+$ cell sorting. (C) Representative flow cytometry data showing the recognition of a HLA-A2 or HLA-B7 (irrelevant)-based dextramer by untransduced, CAR and ΔCAR Tregs. (D) Activation of untransduced, CAR and ΔCAR Tregs following coculture with HLA-A2$^+$ APCs (solid line), HLA-A2$^-$ APCs (dotted line), or no APCs (solid gray). Tregs were cocultured with K562 cells or B-LCLs as APCs for 18 h at a 4:1 Treg:APC ratio after which Treg activation was measured by CD69 expression. K562s were stably transfected to express either HLA-A2 or HLA-A1. SPO (HLA-A2$^+$) and BM21 (HLA-A2$^-$) B-LCLs were used. Data shown are representative of two individual experiments. (E) Pooled flow cytometry data comparing the expression of typical Treg markers by untransduced (black), CAR (light gray), and ΔCAR (dark gray) Tregs. Data shown are mean ± SD pooled from five individual experiments. (F) Pooled flow cytometry data comparing the expression of select skin, gut, and secondary lymphoid organ homing receptors by untransduced (black), CAR (light gray), and ΔCAR (dark gray) Tregs. Data shown are mean ± SD pooled from five individual experiments. (G) Polyclonal suppression assay comparing the suppressive capacity of untransduced (black line), CAR (light gray line), and ΔCAR (dark gray line) Tregs. Tregs and autologous Teffs activated in a polyclonal manner were cocultured at differing ratios for 5 days, after which Teff proliferation was measured by CellTrace Violet dilution. Data are expressed as percentage of inhibition of responder Teff proliferation, relative to Teffs cultured alone. Data shown are mean ± SEM pooled from five to six individual experiments. APCs, antigen-presenting cells; CAR, chimeric antigen receptor; ΔCAR, truncated chimeric antigen receptor; eGFP, enhanced green fluorescent protein; FACS, fluorescence-assisted cell sorting; B-LCL, B-lymphoblastoid cell lines; SD, standard deviation; SEM, standard error of the mean; Teffs, effector T cells; Tregs, regulatory T cells; UN-TDX, untransduced.

This minor increase in suppressive ability may have been due to the antigen-targeting moiety of the ΔCAR facilitating an interaction between the ΔCAR Tregs and the HLA-A2$^+$ APCs, bringing the Treg into the vicinity of the Teff:B-LCL interaction.

Tregs can function by secreting granzyme and perforin (59,60). To exclude the risk of unwanted cytotoxicity, Tregs were cocultured with confluent monolayers of HLA-A2$^+$ and HLA-A2$^-$ epithelial cells, as previously described (Figure 1). Unlike CAR Teffs, CAR Tregs exhibited no detectable level of cytotoxicity toward the HLA-A2$^+$ cells (Figure 3D). IL-2 secretion was undetectable in all conditions analyzed (data not shown) and low levels of IFNγ (221 ± 80 pg/μL; Figure 3E) were produced by CAR Tregs cocultured with HLA-A2$^+$ monolayers. In contrast, these cells produced significantly high levels of the immunosuppressive cytokine IL-10 (1055 ± 78 pg/μL) compared to polyclonal Tregs (not detected) and ΔCAR Tregs (8 ± 1 pg/μL) (Figure 3F), suggesting that *in vivo*, CAR Tregs would contribute to the establishment of an intragraft immunosuppressive milieu in HLA-A2$^+$ allografts.

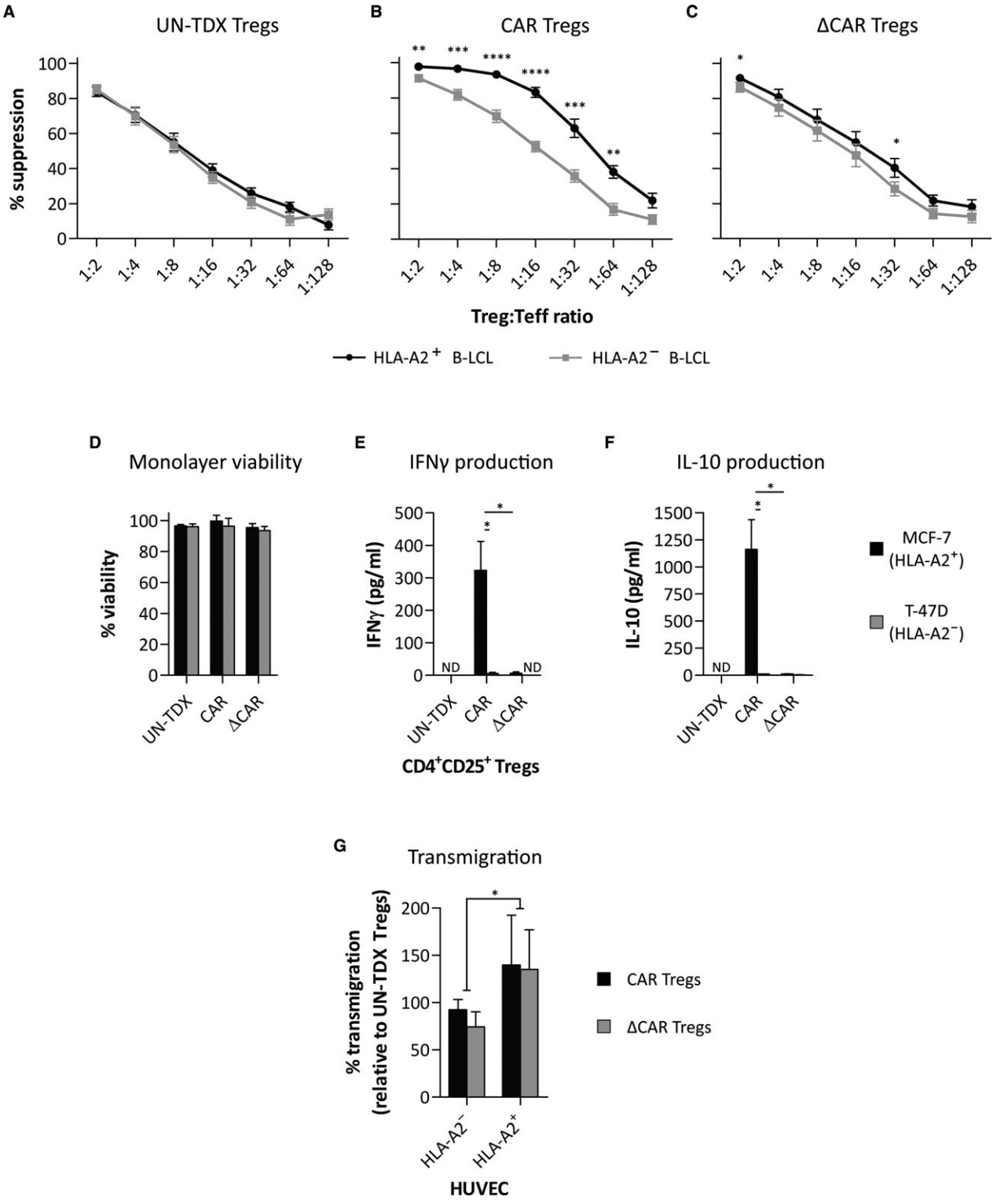
Given the importance of Treg trafficking *in vivo*, we investigated whether the expression of HLA-A2 by endothelial cells influenced the rate with which CAR Tregs transmigrated (61). Relative to polyclonal Tregs, CAR and ΔCAR Tregs transmigrated through HLA-A2$^+$ endothelial monolayers significantly faster than through HLA-A2$^-$ endothelial monolayers ($p = 0.042$; Figure 3G), suggesting a preferential migration into HLA-A2$^+$ target tissues.

Overall, these results demonstrated that compared to polyclonal Tregs, donor-specific CAR Tregs exhibited a greater suppressive capacity in the presence of HLA-A2 without eliciting cytotoxic activity and transmigrated at a faster rate through HUVECs expressing HLA-A2.

CAR-mediated Treg allorecognition conferred a functional advantage in preventing skin graft rejection

We have previously demonstrated the superior efficacy of human Tregs with direct allospecificity over polyclonal Tregs at reducing alloimmune injury in a human skin xenograft transplant model (19,31). To investigate whether CAR Tregs elicited a similar protective role, the same model was employed (Figure S4). Human skin was obtained from routine abdominal angioplasty surgery and HLA-A2 expression was determined using collagenase-treated skin explants (Figure S5). BRG mice were transplanted with HLA-A2$^+$ skin grafts and following skin engraftment, were injected with allogeneic CD25-depleted HLA-A2$^-$ PBMCs ± autologous Tregs. Mice did not exhibit any signs of GvHD throughout the experiments (Figure S6A). Skin grafts were explanted and analyzed histologically for changes in the skin morphology and immune cell infiltration 5 weeks post-PBMC transfer (Figure S4B). Cryosections were stained with hematoxylin and eosin (Figure S6B) or analyzed by immunofluorescence.

Significant alloimmune damage was observed in the grafts of mice that received PBMCs, as demonstrated by a high number of Ki67$^+$ keratinocytes (Figure 4A), a loss of CD31$^+$ blood vessel integrity and loss of the involucrin-expressing epidermal layer (Figure 4B). Grafts of mice treated with PBMCs and Tregs had comparatively fewer Ki67$^+$ keratinocytes (Figure 4A), intact CD31$^+$ blood vessels, and a defined involucrin layer (62) (Figure 4B). Quantification of these observations demonstrated that CAR Treg treatment reduced the number of Ki67$^+$ keratinocytes to basal levels, exhibiting significantly more protection than polyclonal Tregs ($p = 0.042$) (Figure 4D).



Interestingly, in this readout of graft damage, ΔCAR Tregs also mediated superior protection to polyclonal Tregs, although statistical significance was not reached ($p = 0.11$). Conversely, when measuring damage in terms of $CD31^+$ blood vessel integrity, CAR Tregs ($CD31^+$ cluster size = 88.9 ± 8.5; $p < 0.0001$) mediated protection more effectively than both polyclonal (73.3 ± 8.7; $p = 0.017$) and ΔCAR (70.9 ± 10.1; $p = 0.026$) Tregs

Figure 3: Human CAR Tregs functioned more effectively in the presence of HLA-A2 without eliciting cytotoxicity. The suppressive capacity of untransduced (A), CAR (B), and ΔCAR (C) Tregs in the presence of HLA-A2$^+$ B-LCLs (black line) or HLA-A2$^-$ B-LCLs (gray line). Data shown are mean ± SEM pooled from five individual experiments. Significance was determined by two-tailed paired Student's t-test where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$. (D) Quantification of MCF-7 (black) and T-47D (gray) viability following overnight coculture with untransduced, CAR or ΔCAR Tregs as measured by MTT assay. Percent viability is relative to monolayers cultured alone. IFNγ (E) and IL-10 (F) production by the Tregs during coculture with cell monolayers, as measured by ELISA. Data shown are mean ± SEM pooled from three to four individual experiments. Significance was determined by two-tailed paired Student's t-test where * = $p < 0.05$. ND = not detected. (G) Transmigratory capacity of CAR (black) and ΔCAR (gray) Tregs across IFNγ pretreated HLA-A2$^-$ and HLA-A2$^+$ HUVEC endothelial cell monolayers. Data shown represent the percentage of CAR and ΔCAR Tregs that transmigrated, relative to untransduced (UN-TDX) Tregs. Data shown are mean ± SEM pooled from two to six individual experiments. Significance was determined by two-way ANOVA where * = $p < 0.05$. ANOVA, analysis of variance; CAR, chimeric antigen receptor; ΔCAR, truncated chimeric antigen receptor; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical cord endothelial cell; IFNγ, interferon γ; B-LCL, B-lymphoblastoid cell lines; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Teff, effector T cell; Tregs, regulatory T cells.

(Figure 4E). Mice that received PBMCs with CAR or ΔCAR Tregs ($p < 0.0001$ versus PBMCs alone) had a greater FOXP3:CD3 ratio than mice that received PBMCs with polyclonal Tregs ($p = 0.019$). Together, these results suggested that CAR expression facilitated preferential homing and retention of the Tregs in the HLA-A2$^+$ allografts. This localization to the graft enabled the ΔCAR Tregs to elicit more protection than polyclonal Tregs, likely due to these cells being activated in a TCR-dependent manner through direct allorecognition. However, the protection offered was further improved upon by the CAR Tregs that were activated in both a TCR-dependent (direct allorecognition) and CAR-dependent manner.

In conclusion, CAR-engineered HLA-A2-specific Tregs inhibited alloimmune-mediated injury against HLA-A2$^+$ skin allografts significantly more effectively than polyclonal or ΔCAR Tregs, demonstrating the increased potency of these cells *in vivo* and the requirement for Treg signaling to elicit this response.

Discussion

Animal models of transplantation have demonstrated that allospecific Tregs are superior to polyclonal Tregs at protecting from allograft rejection (4,17,19,31–34). Here, we isolated human Tregs using GMP-compatible protocols and used CAR technology to generate donor-MHC class I-allospecific CAR Tregs, which were functionally superior to polyclonal Tregs *in vitro* and in a human skin xenograft transplant model. These results demonstrated a promising new direction for clinical trials that are currently assessing the safety and efficacy of polyclonal Treg therapy in kidney (The ONE Study: NCT02129881) and liver (ThRIL: NCT02166177) transplant recipients (29,30).

Tregs that recognize allo-MHC-peptide complexes (direct allorecognition) and/or allopeptides presented in the context of recipient MHC (indirect allospecificity) have been shown to suppress alloimmune responses more effectively than polyclonal Tregs (4,17–19,31–34). However, the potential of CD4$^+$ Tregs is limited as they are MHC class II-restricted, thus activated primarily by professional APCs. CARs recognize their target antigen in an MHC-independent manner and as such, can confer specificity for donor MHC class I, an alloantigen ubiquitously expressed on tissue parenchyma throughout an allograft.

In this study, we selected HLA-A2 as a target antigen due to its comparatively high prevalence (>40%) in UK donors (63). The scFv used to construct the HLA-A2-specific CAR cross-reacts with HLA-A28 and HLA-A68 (50). CARs that cross-react with various HLA alleles can be used in a wide variety of donor–recipient combinations; thus we believe that generating a library of HLA class I-specific CARs using cross-reactive scFvs will allow for an efficient adaptation into the clinic. Furthermore, we demonstrated that these CARs can be efficiently delivered into human Tregs by lentiviral transduction, which is currently employed clinically to deliver CD19-specific CARs into Teffs for the treatment of CLL (37,38). Progress based on the CRISPR/Cas9 system may make more directed and safer gene delivery accessible in the near future (33).

CAR Tregs demonstrably protected HLA-A2$^+$ human skin grafts more effectively than polyclonal Tregs. However, interesting results were also obtained for the ΔCAR Tregs. These cells exhibited a favored migration and retention in HLA-A2$^+$ target tissues, which enabled the elicitation of greater graft protection than polyclonal Tregs. However, in terms of CD31$^+$ blood vessel integrity, the protection offered by the ΔCAR Tregs was not as great as the CAR Tregs. These findings suggested that the Treg localization was an important factor in determining the protection offered, particularly as TCR-mediated direct allorecognition could facilitate activation of these cells in the graft. However, to exploit the full potential of CAR technology, a functional CAR that activated Tregs in the presence of HLA-A2 was required.

Although CARs have principally been used to generate cancer-specific Teffs, studies investigating the therapeutic potential of CAR Tregs have been performed. Very recently, the efficacy of human CAR Tregs was

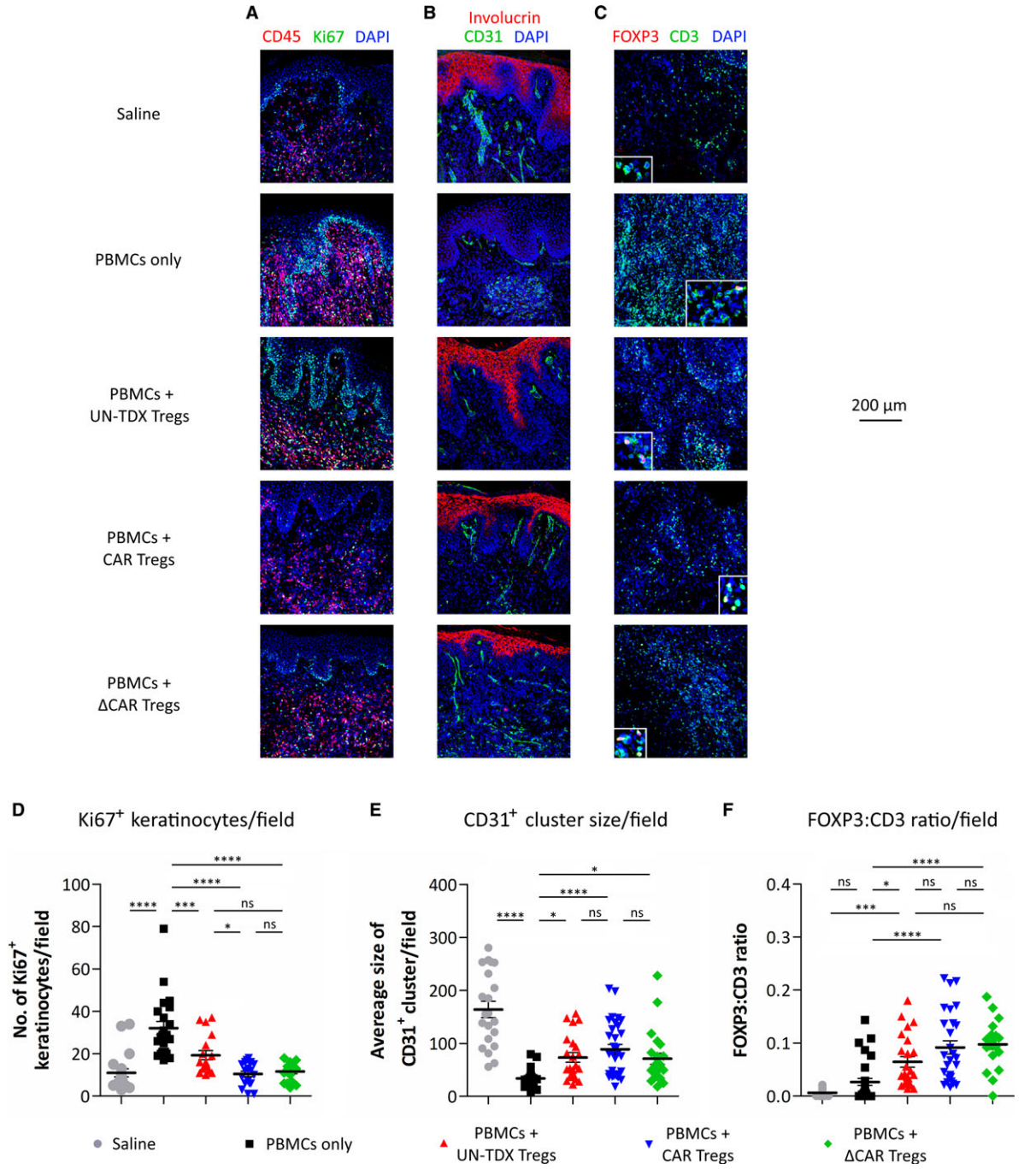


Figure 4: Human CAR Tregs inhibited alloimmune-mediated injury of human HLA-A2$^+$ skin grafts more effectively than polyclonal Tregs. Immunodeficient BRG mice that had received a HLA-A2$^+$ human skin graft were injected with 5×10^6 PBMCs $\pm$ 1×10^6 Tregs. Control mice received saline only. Human skin grafts were removed 5 weeks postinjection and cryopreserved sections were fixed and stained either for human CD45/Ki67/DAPI (A), CD31/involucrin/DAPI (B), or human FOXP3/human CD3/DAPI (C). Images are representative immunofluorescence stains of human skin grafts. Quantification of the number of Ki67$^+$ keratinocytes (D), CD31$^+$ cluster size (E), and FOXP3:CD3 ratio (F) per field of view was performed using NIS Elements and FIJI imaging software (51). Results represent two to three mice/group where four to six fields of view were quantified per section and data are representative of two individual experiments. Significance was determined by one-way ANOVA and the Tukey multiple comparison post-hoc test where * = $p < 0.05$, *** = $p < 0.001$, and **** = $p < 0.0001$. ANOVA, analysis of variance; CAR, chimeric antigen receptor; ΔCAR, truncated chimeric antigen receptor; DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant; PBMCs, peripheral blood mononuclear cells; Tregs, regulatory T cells; UN-TDX, untransduced.

demonstrated in the prevention of xeno-GvHD (49). Furthermore, in preclinical models of colitis (16,44,45) and multiple sclerosis (46), CAR Tregs were found to migrate to locations where their cognate antigen was expressed and suppress undesired immune responses more effectively than polyclonal Tregs. Similarly, we have shown that CAR Tregs redirected toward HLA-A2 preferentially transmigrate through alloantigen-expressing endothelial cells and exhibit a favored homing and retention in allografts. These observations suggest a clinical potential for CAR Tregs outside of the transplant field, particularly in light of ongoing clinical trials that are assessing the safety and efficacy of polyclonal Treg therapy for the treatment of type I diabetes (NCT01210664) (27), lupus erythematosus (NCT02428309), and uveitis (NCT02494492).

In conclusion, polyclonal Treg therapy is currently being investigated clinically by us and others as a means of limiting graft rejection. However, to avoid the risk of pan-immunosuppression and provide a tailored therapy, the successful generation and expansion of alloantigen-specific Tregs is required. We demonstrated that clinically applicable CAR technology may be used to generate donor antigen-specific Tregs that suppress alloimmune responses, providing a future direction for Treg therapy in the pursuit of transplant tolerance in solid organ transplantation.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Isolation purity of human $CD4^+CD25^+$ Tregs. (A) Following isolation, Tregs were costained for CD4 (OKT4) and CD25 (4E3) and the purity was determined by flow cytometry to be 96.2%. Data are representative of 15 individual experiments. (B) Following live/dead discrimination, cells were assessed for expression of the following markers by flow cytometry: CD4 (OKT4), CD25 (4E3), CD127 (A019D5), FOXP3 (PCH101), CTLA-4 (BNI3), and CD39 (eBioA1). Data shown are mean ± standard deviation pooled from six individual experiments.

Figure S2: Proliferation of Tregs in the presence of $HLA\text{-}A2^+$ and $HLA\text{-}A2^-$ APCs. Proliferation of untransduced (red), CAR (blue), and ΔCAR (green) Tregs following coculture with $HLA\text{-}A2^+$ and $HLA\text{-}A2^-$ APCs. Tregs were cocultured with K562 cells (A) or B-LCLs (B) as APCs for 72 hours at a 1:1 ratio. Treg proliferation was measured by ^{3}H-thymidine incorporation and is shown in cpm (counts per minute) where the cpm of APCs cultured alone was subtracted. K562s were stably transfected to express either HLA-A2 or HLA-A1. SPO ($HLA\text{-}A2^+$) and BM21 ($HLA\text{-}A2^-$) B-LCLs were used. Data shown are mean ± standard deviation and are representative of two individual experiments. Significance was determined by two-tailed paired Student's t-test where * = $p < 0.05$. ns, not significant.

Figure S3: Detailed phenotypic analysis of transduced human Tregs. Representative flow cytometry plots comparing the expression of typical Tregs markers (A) and various homing receptors (B) by untransduced (UN-TDX; red line), CAR (blue line), and ΔCAR (green line) Tregs. Isotype control staining is shown in solid gray. Data shown are representative of five individual experiments.

Figure S4: Schematic diagrams detailing the experimental design of the human skin xenograft transplant experiments. (A) Human skin from $HLA\text{-}A2^+$ donors was transplanted onto recipient immunodeficient BRG mice and allowed to engraft for 5–6 weeks. These mice were then injected intravenously with allogeneic $HLA\text{-}A2^-$ PBMCs with or without autologous *ex vivo*-expanded Tregs. Mice were sacrificed 5 weeks following PBMC infusion and skin grafts were monitored histologically for changes in skin morphology and cell infiltration. (B) Timeline showing when mice were transplanted with human skin, injected with allogeneic PBMCs ± Tregs, and sacrificed to analyze skin graft histology.

Figure S5: HLA-A2 expression on skin-derived cells. Small explants of human skin were tested for HLA-A2 expression by flow cytometry prior to transplantation. Cells acquired by treating explants with collagenase for 1 hour were stained with two separate HLA-A2-specific antibody clones (BB7.2 and REA142 denoted by blue and green lines, respectively). HLA-A2-expression was compared to cells stained with an isotype control, shown in solid gray.

Figure S6: Mice transplanted with human skin allografts did not lose weight following PBMC transfer. (A) Transplanted mice injected with allogeneic PBMCs ± Tregs showed no signs of graft-versus-host disease and no weight loss. (B) Representative hematoxylin/eosin stains of skin grafts stained in Figure 4.

"Finally, from so little sleeping and so much reading, his brain dried up and he went completely out of his mind."

Don Quixote de la Mancha, Miguel de Cervantes Saavedra